REMARKS

Claims 11, 12 and 15-38 were pending in the subject application. Applicants have hereinabove amended claims 11 and 12. Support for the amendment to the claims may be found, *inter alia*, in the specification at page 3, lines 4-21. Applicants maintain that no issue of new matter is raised by this amendment. Upon entry of the Amendment, claims 11, and 12, as amended, and claims 15-38 will be pending and under examination.

The Examiner stated that applicants should update the status of the instant case at page 1, line 9, of the instantly filed specification to state that case serial number 10/079,038, filed February 20, 2002, which the present case is a continuation of, has issued as U.S. Patent No. 6,627,623. Applicants have amended the specification to include the patent number and issue date of case serial number 10/079,038.

Rejection under 35 U.S.C. §112, First Paragraph

The Examiner rejected claims 11, 12 and 15-38 under 35 U.S.C. §112, First Paragraph, as failing to comply with the enablement requirement. The Examiner stated that the claims contain subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. The Examiner stated that the claims were broadly drawn to methods of treating cancer. The Examiner admits that the specification teaches a variety of basic experimental analysis to illustrate the treatment of cancer in vitro of breast cancer cells, prostrate cancer cells, ovarian cancer cells, etc. and further includes the *in-vitro* induction of phosphorylation of Bcl-2. However, the Examiner states that with regards to decreasing cell vitality in a dose-dependent manner, the specification only teaches in vitro experimental analysis demonstrating the decrease in cell vitality and provides no extrapolation of data to support in vivo decrease of cell vitality with respect to cancer cells

of the breast, prostrate, ovarian, etc.

In response, applicants respectfully traverse the Examiner's rejection. However, in order to expedite prosecution and without conceding the correctness of the Examiner's position, applicants have hereinabove amended independent claims 11 and 12 to recite, in relevant part, that the cancer is breast, prostate or ovarian cancer.

Applicants note that the tested cell models in the specification are for breast, prostate and ovarian cancers (e.g. MCF-7, PC-3, Du-145 and PA-1 cells). See, *inter alia*, page 3, lines 7-10 and 14; page 5, lines 16-22. These are standard models for their corresponding cancers. *See* Vickers, et al., 1988; Pink, et al., 1996; Zhang, et al., 2003; Mora, et al., 2002; Ma, et al., 1988; and Obermiller, et al., 1999, attached hereto as Exhibits 1-6, respectively. Moreover, MPEP 2164.02 notes that the Examiner must show "the model" itself does not correlate. The Examiner's general statements regarding in vitro/in vivo correlation do not meet the required standard. In addition, MPEP 2164.02 makes it clear that a "rigorous or an invariable exact correlation" is <u>not</u> required. Accordingly, one of skill in the art, in view of the art's recognition of the models, would expect correlation between the successful in vitro induced BCL-2 phosphorylation as observed in the specification and treatment of the specific corresponding cancer, absent evidence to the contrary. See, *inter alia*, page 1, line 21 - page 2, line 25; page 3, lines 7-21. Furthermore, one of skill in the art of clinical studies would well be able to determine dosage ranges for any animal, for any route of administration, by treating test animals and biopsying their tumor cells and measuring the level of Bcl-2 phosphorylation in those tumor cells as described at page 6, lines 6-18 of the specification, for the methods as now claimed. Moreover, such a dose-finding study is routine in the clinical studies arts and is not undue experimentation. Accordingly, one of skill in the art would be able to make and/or use the invention without undue experimentation.

Applicants submit that, in view of the preceding remarks, claims 11 and 12 meet the requirements of 35 U.S.C. §112, First Paragraph, and are enabled for the reasons stated hereinabove. Claims 15-38 depend from either claim 11 or 12 and are submitted to be enabled for the same reasons. Applicants therefore respectfully request that the Examiner reconsider and withdraw the rejection of claims under 35 U.S.C. §112, First Paragraph.

Summary

In view of the remarks hereinabove, applicants respectfully submit that the grounds of rejection set forth in the April 29, 2008 Office Action have been overcome. Applicants therefore respectfully request that the Examiner reconsider and withdraw these grounds of rejection and indicate that the claims are allowable.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicant's undersigned attorney invites the Examiner to telephone him at the number provided below.

No fees, other than the enclosed $555.00 three-month extension fee, is deemed necessary in connection with the filing of this Amendment. Accordingly, a check in the amount of $555.00 is enclosed. However, if any additional fee is required, authorization is hereby given to charge the amount of such fee to Deposit Account No. 03-3125.

Respectfully submitted,

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Cooper & Dunham LLP
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John P. White — Date
Reg. No. 28,678

MOLECULAR ENDOCRINOLOGY

Molecular Endocrinology, Vol 2, 886-892, Copyright © 1988 by Endocrine Society

ARTICLES

A multidrug-resistant MCF-7 human breast cancer cell line which exhibits cross-resistance to antiestrogens and hormone-independent tumor growth in vivo

PJ Vickers, RB Dickson, R Shoemaker and KH Cowan
Clinical Pharmacology Branch, National Cancer Institute, Bethesda, Maryland 20892.

MCF-7 human breast cancer cells provide a useful in vitro model system to study hormone-responsive breast cancer as they contain receptors for estrogen and progesterone, and estrogen both induces the synthesis of specific proteins in these cells and increases their rate of proliferation. An MCF-7 cell line which was selected for resistance to adriamycin (MCF-7/AdrR) exhibits the phenotype of multidrug resistance (MDR), and displays multiple biochemical changes. MDR in MCF-7/AdrR is also associated with a loss of mitogenic response to estrogen and the development of cross-resistance to the antiestrogen 4-hydroxytamoxifen. In addition, while the parental MCF-7 cell line responds to estrogen with increased levels of progesterone receptors and the secretion of specific proteins, these estrogen responses are lost in MCF-7/AdrR. Furthermore, while the formation of tumors in nude mice by wild-type MCF-7 cells is dependent upon the presence of estrogen, MCF-7/AdrR cells form tumors in the absence of exogenous estrogen administration. These changes in hormonal sensitivity and estrogen-independent tumorigenicity of the multidrug-resistant MCF-7 cell line are associated with a loss of the estrogen receptor and a concomitant increase in the level of receptors for epidermal growth factor. Thus, in MCF-7/AdrR cells, the development of MDR is associated with alterations in the expression of both cytosolic and membrane receptors, resulting in resistance to hormonal agents and the expression of hormone-independent tumor formation.

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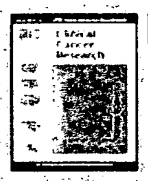
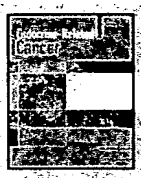
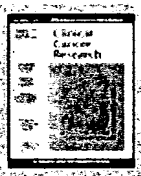
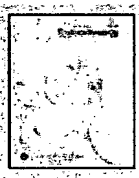




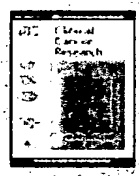




[Abstract] [Full Text] [PDF]

MOLECULAR ENDOCRINOLOGY ▸HOME

A. S. Oh, L. A. Lorant, J. N. Holloway, D. L. Miller, F. G. Kern, and D. El-Ashry
Hyperactivation of MAPK Induces Loss of ER{alpha} Expression in Breast Cancer Cells
Mol. Endocrinol., August 1, 2001; 15(8): 1344 - 1359.
[Abstract] [Full Text] [PDF]



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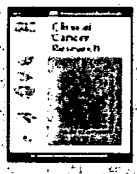
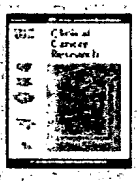
C. Qin, C. Wilson, C. Blancher, M. Taylor, S. Safe, and A. L. Harris
Association of ARNT Splice Variants with Estrogen Receptor-negative Breast Cancer, Poor Induction of Vascular Endothelial Growth Factor under Hypoxia, and Poor Prognosis
Clin. Cancer Res., April 1, 2001; 7(4): 818 - 823.
[Abstract] [Full Text]



Clinical Cancer Research ▸HOME

C. Bianco, R. Bianco, G. Tortora, V. Damiano, P. Guerrieri, P. Montemaggi, J. Mendelsohn, S. De Placido, A. R. Bianco, and F. Ciardiello
Antitumor Activity of Combined Treatment of Human Cancer Cells with Ionizing Radiation and Anti-Epidermal Growth Factor Receptor Monoclonal Antibody C225 plus Type I Protein Kinase A Antisense Oligonucleotide
Clin. Cancer Res., November 1, 2000; 6(11): 4343 - 4350.
[Abstract] [Full Text] [PDF]



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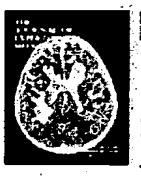
F. Ciardiello, R. Bianco, V. Damiano, S. De Lorenzo, S. Pepe, S. De Placido, Z. Fan, J. Mendelsohn, A. R. Bianco, and G. Tortora
Antitumor Activity of Sequential Treatment with Topotecan and Anti-Epidermal Growth Factor Receptor Monoclonal Antibody C225
Clin. Cancer Res., April 1, 1999; 5(4): 909 - 916.
[Abstract] [Full Text] [PDF]



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Defective Acidification in Human Breast Tumor Cells and Implications for Chemotherapy
J. Exp. Med., May 18, 1998; 187(10): 1583 - 1598.
[Abstract] [Full Text] [PDF]

[CANCER RESEARCH 56, 2321–2330, May 15, 1996]

Models of Estrogen Receptor Regulation by Estrogens and Antiestrogens in Breast Cancer Cell Lines[1]

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Department of Human Oncology, University of Wisconsin Comprehensive Cancer Center, Madison, Wisconsin 53792 [J. J. P., V. C. J.], and Robert H. Lurie Cancer Center, Northwestern University Medical School, Chicago, Illinois 60611 [V. C. J.]

ABSTRACT

The expression and stability of the estrogen receptor (ER) is the result of a complex process that is modulated by estrogens and antiestrogens. Regulation of the steady-state ER mRNA and protein levels in breast cancer cells appears to be the result of either of two distinct regulatory mechanisms. Estrogen exposure causes a rapid down-regulation of the steady-state level of ER mRNA and protein in model I regulation, as exemplified by the MCF-7:WS8 cell line. Conversely, in model II regulation, as observed in the T47D:A18 cell line, estrogen exposure causes an increase in the steady-state ER mRNA level and a maintenance of the ER protein level. In both these cell lines, the nonsteroidal antiestrogen 4-hydroxytamoxifen has little effect on the mRNA level but causes a net accumulation of the ER protein over time. In contrast, the pure antiestrogen ICI 182,780 causes a dramatic reduction of the ER protein in both the MCF-7:WS8 and T47D:A18 cell lines. This loss has little effect upon the ER mRNA level in the MCF-7:WS8 cells but leads to a decline in the ER mRNA in the T47D:A18 cells. The estrogen-independent MCF-7:2A cell line, which has adapted to growth in estrogen free media, expresses two forms of the ER, a wild-type M_r 66,000 ER and a mutant M_r 77,000 ER (ER^{77}). ER^{77} is the product of a genomic rearrangement resulting in a tandem duplication of exons 6 and 7 (J. J. Pink *et al.*, Nucleic Acids Res., 24: 962–969, 1996). This exon duplication has abolished ligand binding by this protein. Here we demonstrate that the loss of ligand binding has eliminated the effects of 4-OHT and ICI 182,780 on the steady-state ER^{77} protein level. However, in the MCF-7:2A cells, antiestrogens affect the wild-type ER protein in the same manner as observed in the MCF-7:WS8 and T47D:A18 cells. Estrogen regulates the ER mRNA and wild-type ER and ER^{77} proteins in the MCF-7:2A cells in the same manner as observed in the MCF-7:WS8 cells. Interestingly, treatment of the MCF-7:2A cells with ICI 182,780 causes a slight increase in ER mRNA, which is reflected in a net increase in the ER^{77} protein but a dramatic decrease in the wild-type ER. The models presented here describe the response of two human breast cancer cell lines in short-term studies. These distinct regulation pathways are predictive of the response of these cell lines to long-term estrogen deprivation. This study illustrates two alternative regulation pathways that are present in ER-positive, estrogen-dependent breast cancer cells. This variable response highlights the diversity of responses potentially present in the heterogeneous cell populations of clinically observed breast cancer.

INTRODUCTION

The treatment and prognosis of breast cancer has been improved dramatically during the past decade through the use of the nonsteroidal antiestrogen tamoxifen (1, 2). Although it has long been known that tamoxifen and its metabolites compete with estrogens for binding to the ER,[3] the downstream effects of this binding are unclear. The most active metabolite of tamoxifen, which is used in all studies described here, is 4-OHT, which has a binding affinity for the ER 50–100-fold greater than that of tamoxifen (3). The continuing search for new and more effective antiestrogens lead to the synthesis of the 7α-substituted estradiol analogues such as ICI 164,384 and ICI 182,780 (4). These compounds are classified as pure antiestrogens due to their complete lack of any estrogenic activities and are currently being evaluated in clinical trials for the treatment of tamoxifen-resistant breast cancer (5). In contrast to the pure antiestrogens, the nonsteroidal antiestrogens such as tamoxifen display some estrogenic characteristics, depending upon the species and tissue type tested, and are, therefore, classified as partial agonists (6). In addition to the effects these compounds have on the function of the ER, it has been known for many years that estrogens and antiestrogens can regulate the steady-state level of the ER (7–27).

Since the isolation and characterization of the MCF-7 cell line in 1973, ER-positive breast cancer cells lines have become the standard laboratory model in which to study the effects of estrogens and antiestrogens on breast cancer (28–31). The use of these valuable models throughout the past decade has lead to the evaluation of the effects of estrogens and antiestrogens in various cell culture systems (31–35). Previous studies have demonstrated the mechanism by which estrogens and pure antiestrogens regulate ER protein to be quite different. Both compounds cause a significant decrease in the steady-state level of the ER in specific cell lines (15, 18). Estrogens function by initially decreasing transcription of the ER and later by destabilizing the ER mRNA (15, 24). The pure antiestrogens, in contrast, have little effect on the transcription of the ER and function by directly causing a rapid degradation of the ER protein (36, 37) This degradation appears to be the result of an alteration of the nucleoplasmic shuttling of the receptor (38).

Regrettably, only a few different ER-positive cell lines are available in which to study the effects of estrogens and antiestrogens on the growth and gene expression in breast cancer cells. These represent only a few patients from which to generalize the findings. The standard cell line MCF-7 is dependent upon the presence of estrogens for maximal growth, and this growth can be competitively inhibited by antiestrogens. In virtually all published reports, E_2 has been shown to lead to a rapid reduction in the steady-state levels of ER mRNA and protein in MCF-7 cells (15, 18, 39–41).

Another well-studied human breast cancer cell line, T47D, exhibits a different phenotype. The growth dependence and ER and PR expression of the various T47D clones used throughout the world reveals a remarkable diversity. The T47D cell line was originally characterized as an ER- and PR-positive, estrogen-dependent cell line (42). In the many years since it's initial characterization, the cells have diverged and given rise to clones with very different growth and expression phenotypes. T47D lines have been described which range from completely estrogen independent to classically estrogen dependent (43–48). The clone that is currently used in our laboratory,

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[2] To whom requests for reprints should be addressed, at Robert H. Lurie Cancer Center, Northwestern University Medical School, 303 East Chicago Avenue, Olsen Pavilion 8258, Chicago, IL 60611.

[3] The abbreviations used are: ER, estrogen receptor; ER^{77}, mutant M_r 77,000 ER; ERE, estrogen response element; PR, progesterone receptor; E_2, 17β-estradiol; 4-OHT, 4-hydroxytamoxifen.

T47D:A18, requires estrogen for maximal growth, and this estrogen-stimulated growth can be inhibited by antiestrogens (32, 49). The regulation of PR expression in T47D cells, which is considered to be a marker of a functioning ER-mediated transcription pathway (50), has also been shown to be variable depending on the source of the cells. Some groups find that the PR is constitutively expressed and others, including our laboratory, find that the expression of the PR is regulated by estrogens. In T47D cells, the regulation of ER mRNA has been reported to be different than that observed in MCF-7 cells, with estrogen exposure giving rise to an increase in ER mRNA (18). For our studies, we classify the T47D:A18 cell line as ER positive and estrogen dependent.

To study the changes associated with the development of estrogen-independent growth we have developed a subclone of MCF-7 which now grows maximally in the absence of estrogens. This clonal cell line, named MCF-7:2A, was derived by growing MCF-7 cells in estrogen-free media for 8 months, followed by two rounds of limiting dilution cloning (51). In addition to being estrogen independent, these cells also express a novel M_r 77,000 ER. This ER, which is referred to as ER^{77}, is the result of a genomic rearrangement which has lead to a tandem duplication of exons 6 and 7 in the *ER* gene.[4] This mutation has abrogated all ligand binding by ER^{77}. The wild-type M_r 66,000 ER is also expressed in these cells at a 4–10-fold higher level than the ER^{77}. This cell line provides us with a unique model in which to study the regulation of two ERs with dramatic functional differences in a single cell line.

We believe that it is important to examine the differential effects of estrogens and different classes of antiestrogens on ER regulation to establish a clear understanding of this valuable therapeutic target. We have compared and contrasted the effects of compounds on three different cell lines and used two types of receptor proteins as markers of activity. This evaluation of responses to the diverse actions of these compounds in a single series of experiments gives a unique portrayal of the mechanism of action of estrogens and antiestrogens in human breast cancer cells. Our study reveals that distinct ER-dependent regulatory mechanisms are present in these breast cancer cells.

MATERIALS AND METHODS

Tissue Culture. MCF-7 cells were obtained from Dean Edwards (at the San Antonio Breast Cancer Group, TX; originally obtained from the Michigan Cancer Foundation Detroit, MI). T47D (42) cells were obtained from American Type Culture Collection (Rockville, MD). MCF-7:2A cells were derived from the MCF-7 line by growth in estrogen-free media and two rounds of limiting dilution cloning (51). All tissue culture components were obtained from Life Technologies, Inc. (Grand Island, NY) unless otherwise stated. MCF-7:WS8 and T47D:A18 cells were grown in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (Bioproducts for Science Inc., Indianapolis, IN), 6 ng/ml bovine insulin, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (fully estrogenized medium). MCF-7:2A cells were routinely grown in estrogen-free medium that substitutes phenol red-free RPMI and 3× dextran-coated, charcoal-treated fetal bovine serum. Cells were passed at 1:10–1:20 dilutions once per week using 0.1% trypsin.

Growth Assays. All cells were grown in estrogen-free media for at least 2 days prior to the beginning of each experiment. Cells were seeded into each well of a 24-well plate (20,000 cells/well) in 1 ml of estrogen-free media on day 0. The following day (day 1), this media was removed, and 1 ml of media containing the appropriate compound was added. All compounds were dissolved in 100% ethanol and added to media at a 1:1000 dilution. Media was changed on day 4, and experiments were ended on day 6. DNA content was determined by the method of LaBarca and Paigan (52) using an fluorocolorimeter II (SLM Aminco, Urbana, IL). 17β-Estradiol was purchased from Sigma Chemical Corp., 4-OHT was a generous gift from Zeneca Pharmaceuticals (Macclesfield, England), and ICI 182,780 was a generous gift from Dr. Alan Wakeling (Zeneca Pharmaceuticals).

Transient Transfection Assays. Cells were seeded into a 6-well plate (500,000 cells/well) in phenol red-free RPMI plus 10% 3× charcoal-stripped fetal bovine serum. The following day, medium was removed and replaced with fresh estrogen-free medium. A solution containing 1 μg of the luciferase reporter construct pVIT3-luc (51) and 0.5 μg of the β-galactosidase reporter pCMVβ (53) in 0.25 M $CaCl_2$ was mixed dropwise with an equal volume of 2× HBS (0.28 M NaCl, 0.05 M HEPES, and 1.5 mM $NaPO_4$, pH 7.05) by gently bubbling air through the solutions. This solution was then incubated at room temperature for 20 min to allow a DNA/$CaPO_4$ precipitate to form. This solution (0.4ml) was slowly added to the cells in 3.6 ml media and incubated at 37°C in a humidified incubator with 5% CO_2 for 6 h. At that time, the DNA solution was removed, and the cells were shocked with a solution of 10% glycerol in 1× HBS for 3 min. This solution was then removed, and the cells were washed 2× with 4 ml PBS. Medium with or without compounds was then added to the wells and incubated at 37°C in a humidified 5% CO_2 incubator for an additional 42 h. The media was removed, and the cells were washed once with ice-cold PBS. The cells were then scraped in extraction buffer [0.1 M $KHPO_4$ (pH 7.5), 1% Triton X-100, 100 μg/ml BSA, 2.5 mM phenylmethylsulfonyl fluoride, and 1 mM DTT] and pipetted vigorously to ensure complete cell lysis. Debris was then pelleted by spinning in a microfuge for 1 min, and the lysate was stored on ice until luciferase activity was assayed. Luciferase activity was assayed by mixing 50 μl of each lysate with 350 μl of reaction buffer [160 mM $MgCl_2$, 75 mM glycylglycine (pH 7.8), 0.5 mg/ml BSA, 19 mg/ml ATP, and 15 mM Tris-HCl, pH 7.5]. To begin each assay, 100 μl of substrate (0.4 mg/ml luciferin potassium salt in 10 mM Na_2CO_3, pH 6.0) was automatically injected into the lysate mixture. Each point was monitored for 10 s by a Monolight 2010B luminometer (Analytical Luminescence Laboratory, San Diego, CA), and relative luciferase units were then reported. All points were corrected for transfection efficiency by dividing relative luciferase units by β-galactosidase activity. β-Galactosidase activity was measured using a β-methylumbelliferone assay (54). Briefly, an aliquot on the cell extract was mixed with 1.3 ml reaction buffer containing 0.1 M $NaPO_4$, 10 mM KCl, 1 mM $MgSO_4$ (pH 7.0) and 2.2 × 10^{-5} g/ml β-methylumbelliferone, (Molecular Probes Inc., Eugene, OR). The sample was incubated at room temperature for 1 h, and 750 μl of stop buffer (15 mM EDTA and 0.3 M glycine, pH 11.2) was added. The samples were then read in a LS-5 fluorescence spectrophotometer (Perkin-Elmer, Foster City, CA) with excitation at 350 nm and absorption at 450 nm. All samples were correlated to a standard curve using purified β-galactosidase (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Gel Shift Assays. Nuclear extracts were prepared from cells grown in estrogen-free media for 4 days, followed by 24 h in either control estrogen-free media or media containing 1 μM ICI 182,780. Extracts were prepared by homogenization in 10 mM Tris-HCl (pH 7.5), 400 mM KCl, 10% glycerol, and 1 mM DTT. Gel shift assays were performed using the components provided in the BandShift Kit (Pharmacia Biotech, Piscataway, NJ). All binding reactions contained 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 3 mM DTT, 10% glycerol, 0.05% NP40, 0.1 mM $ZnCl_2$, 50 μg/ml poly(dI·dC), and 1 ng of labeled oligonucleotide. Oligonucleotides were labeled with Klenow polymerase. Compounds were added directly to the reaction mixtures (1 μl of a 20× stock solution in 20% ethanol) and preincubated at room temperature for 15 min, at which time the radiolabeled oligonucleotide was added; then the incubation was continued for an additional 30 min. The samples were placed on ice for 15 min and then separated using nondenaturing PAGE (4%) using 1× low ionic strength buffer [7 mM Tris-HCl (pH 7.5 at 22°C), 3 mM sodium acetate, and 1 mM EDTA) with constant buffer recirculation at 4°C. Following electrophoresis, the gel was dried and exposed to X-ray film.

Western Blotting. For Western blots 1 × 10^5 cells were seeded into a 10-cm tissue culture dish in estrogen-free media. The following day, media containing the appropriate compound was added, and cells were harvested at the indicated times. Whole-cell extracts were prepared by direct lysis of PBS washed cells in sample buffer [10% glycerol, 150 mM Tris-HCl (pH 6.8), 0.5 mM EDTA, 0.125% SDS, 1% β-mercaptoethanol, and 5 μg/ml bromophenol blue], followed by immersion in a boiling water bath for 5–10 min. Equal amounts of protein as determined in a Bio-Rad protein assay were run in a

[4] J. J. Pink, M. Fritsch, M. M. Bilimoria, V. J. Assikis, and V. C. Jordan. Cloning and characterization of a 77 kilodalton estrogen receptor isolated from a human breast cancer cell line, submitted for publication.

standard Western blot as described previously (51) with the following changes. The secondary antibody used was a horseradish peroxidase-conjugated goat antirat antibody (HyClone Laboratories, Logan, UT), and visualization was accomplished using the ECL visualization kit (Amersham Corp., Arlington Heights, IL) as per the manufacturer's directions. The membrane was then wrapped in plastic film and exposed to Kodak X-OMAT film for 15 s to 5 min and developed. Quantitation was performed using a Gel Doc 1000 (Bio-Rad, Hercules, CA).

Northern Blot Analysis. For Northern blotting, 5×10^6 cells were seeded into a 100-cm^2 dish and allowed to attach overnight. The following day, medium containing the appropriate compound was added, and the mRNA was isolated using a procedure of direct poly(A)$^+$ RNA purification at the indicated times (55). The mRNA was denatured by heating to 65°C for 15 min in 10 mM 4-morpholinepropanesulfonic acid (pH 7.0), 4 mM sodium acetate, 0.5 mM EDTA, 6.5% formaldehyde, and 50% deionized formamide. The denatured mRNA was loaded onto a 1.2% agarose/formaldehyde gel and run overnight at 25V with buffer recirculation. Transfer to Hybond N (Amersham Corp., Arlington Heights, IL) was performed using a Vacu-Gene transfer apparatus (Pharmacia Biotech, Inc., Piscataway, NJ) according to the manufacturer's directions. The membranes were then UV fixed using a UV Stratalinker 2400 (Stratagene, La Jolla, CA) and air dried prior to prehybridization. Prehybridization was performed at 47°C using a solution comprised of 5× SSC, 20 mM NaPO4 (pH 6.5), 0.6% polyvinylpyrrolidone, 0.1% Ficoll, 0.1% BSA, 0.2% SDS, 250 μg/ml denatured salmon sperm DNA, 50% deionized formamide, and 10% sodium dextran sulfate. The DNA probes were prepared by random primer labeling using Klenow polymerase (Promega, Madison, WI). Hybridization was carried out by adding $2–4 \times 10^7$ dpm/ml of the denatured probes directly to the prehybridization buffer and incubating for 12–16 h at 47°C. The membranes were then washed in 2× SSC, 0.2% SDS at room temperature for 2–3 h at room temperature with four buffer changes, followed by one wash in 0.1× SSC, 0.2% SDS at 65°C for 15 min. The membranes were then exposed to Kodak X-OMAT film in an autoradiography cassette containing double Quanta III intensifying screens at −70°C for 24–200 h. Quantitation was performed using multiple exposures in a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). All values were corrected by reprobing the blots with β-actin following probing with the human ER cDNA; these corrected values were then compared with matched control samples, and this value is reported as fold control in Figs. 5–7.

RESULTS

Growth Effects of Estrogens and Antiestrogens. The effect of E_2 or the antiestrogens 4-OHT and ICI 182,780 on the growth of three human breast cancer cell lines was measured in a 6-day assay (Fig. 1). In the estrogen-dependent MCF-7:WS8 and T47D:A18 cell lines, growth in estrogen-free media was significantly less than that of the estrogen-independent MCF-7:2A cell line. The addition of 1 nM E_2 to the culture media caused a 5–7-fold increase in the growth of both the MCF-7:WS8 and T47D:A18 lines, as expected; however, E_2 caused an ~20% decrease in the growth of the MCF-7:2A cells. The partial agonist 4-OHT caused a slight increase in the growth of the MCF-7:WS8 and T47D:A18 cells in the absence of estrogens, but this same concentration of 4-OHT inhibited E_2-stimulated growth (data not shown). In the T47D:A18 and MCF-7:WS8 cell lines, the pure antiestrogen ICI 182,780 lead to growth rates that were indistinguishable from that in control media. In the MCF-7:2A cells, both antiestrogens inhibited growth below that in control media, with ICI 182,780 showing slightly more repression that 4-OHT.

Effects on DNA Binding. The effect of estrogen and antiestrogen on the specific DNA binding of the ERs isolated from the MCF-7:WS8 and MCF-7:2A cells was measured in a gel-shift assay (Fig. 2). Cells were grown in estrogen-free media for 4 days, after which the media was replaced with either fresh estrogen-free media or media containing 1 μM ICI 182,780. Nuclear extracts were isolated 24 h following this media change. The ability of the ER in these extracts to bind to a radiolabeled 20-bp oligonucleotide containing the consensus vitellogenin A_2 ERE was then measured in a standard gel-shift assay. The addition of estrogens or antiestrogens to the extracts from estrogen-free cells caused an increase in specific DNA binding as compared to the control groups. The binding was increased equally in all treatment groups, including the ICI 182,780 group. In contrast, the extracts isolated from cells treated for 24 h with ICI 182,780 demonstrated no measurable binding in any group. This finding is consistent with the data from the Western analyses (see below), which demonstrated the rapid degradation of the ER following ICI 182,780 treatment in intact cells. This degradation was not apparent in extracts exposed to ICI 182,780 *in vitro*.

ER Transcriptional Activation. The transcriptional activity of the ER in these cell lines was measured in a luciferase assay. The luciferase reporter construct pVIT3-Luc was used to measure the ability of the ER from these three cell lines to induce transcription following estradiol exposure. pVIT3-Luc contains three copies of the consensus *Xenopus* vitellogenin A_2 ERE upstream of a herpes simplex virus thymidine kinase minimal promoter, which controls transcription of a firefly luciferase reporter gene. This construct is exquisitely sensitive to the activity of the ER, while showing no activity in ER-negative cells (51, 56). As shown in Fig. 3, E_2 induced a dose-dependent increase in the transcription of this reporter gene in all three

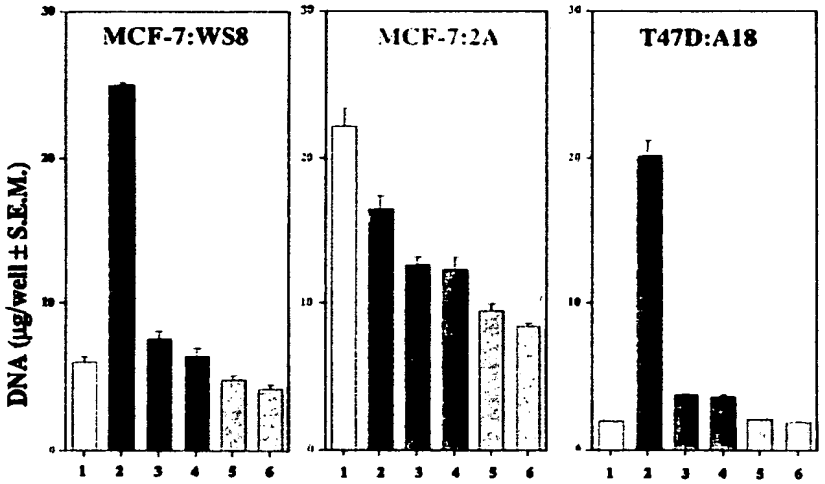


Fig. 1. Growth response of MCF-7:WS8, MCF-7:2A, and T47D:A18 cells to estrogen and antiestrogens. Cells were grown in estrogen-free media for 4 days prior to the start of the experiment. Cells were seeded at 15,000 cells/well in a 24-well plate for 1 day in control media. Medium containing the appropriate compound was added on day 1 and changed on days 3 and 5; then the cells were harvested on day 6. *Lane 1*, Control; *Lane 2*, 1 nM E_2; *Lane 3*, 0.1 μM 4-OHT; *Lane 4*, 1 μM 4-OHT; *Lane 5*, 0.1 μM ICI 182,780; *Lane 6*, 1 μM ICI 182,780. Each column represents the mean of triplicate determinations; *bars*, SE.

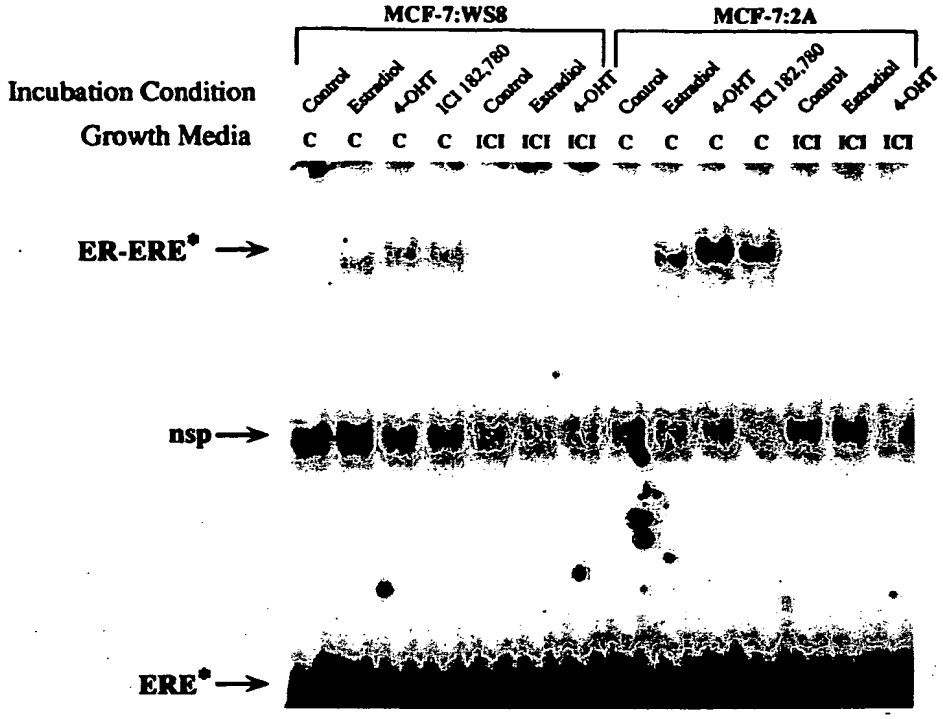


Fig. 2. Gel-shift assay of nuclear extracts from MCF-7:WS8 and MCF-7:2A cells. Nuclear extracts were isolated from MCF-7:WS8 or MCF-7:2A cells following growth for 4 days in estrogen-free media followed by 24 h in control (*C*) or 1 μM ICI 182,780 (*ICI*) containing growth media. Equal amounts of each lysate were then preincubated in binding reactions containing ethanol vehicle only (*Control*), 1 nM E_2 (*Estradiol*), 1 μM 4-OHT, or 1 μM ICI 182,780 as described in "Materials and Methods." Binding to a 20-bp ^{32}P-labeled oligonucleotide containing the vitellogenin A_2 ERE was assessed by electrophoresis in a 4.5% nondenaturing polyacrylamide gel.

cell lines. This activity was inhibited by either 4-OHT or ICI 182,780 in all cells lines (data not shown). The higher basal level seen in the MCF-7:2A cells was reproducible, and this basal expression could be returned to the level observed in the control MCF-7:WS8 cells by the pure antiestrogen ICI 182,780 (51). The administration of ICI 182,780 to the MCF-7:WS8 or T47D:A18 cells did not alter the luciferase expression as compared to the control level (data not shown).

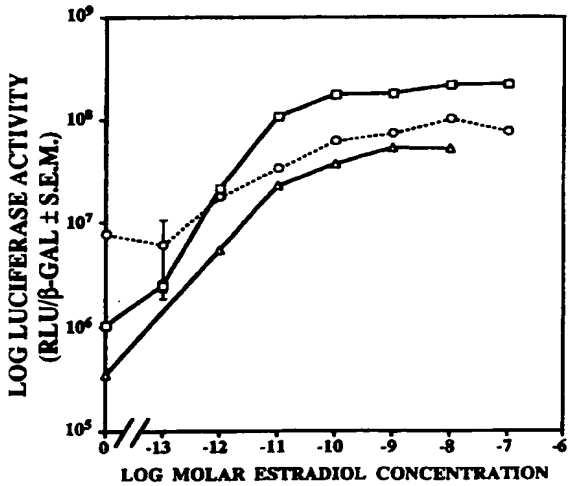


Fig. 3. Induction of luciferase in an estrogen-responsive reporter construct. MCF-7:WS8 (□), T47D:A18 (△), and MCF-7:2A (○) cells were grown for 1 day in estrogen-free media prior to transfection. Transfections were performed using standard calcium phosphate methodology and included 1 μg of the reporter pVIT3-Luc and 0.5 μg of pCMVβ as a transfection control as described in "Materials and Methods." Six h following transfection, the cells were glycerol shocked, and media containing the denoted concentration of E_2 was added. Cells were harvested, and luciferase activity was determined 42 h following the addition of compound. All values are the mean of duplicate determinations, which have been corrected for β-galactosidase activity.

Regulation of Steady-State ER mRNA and Protein. The effect of E_2 and two types of antiestrogens on the steady-state level of ER mRNA was assessed by quantitation of Northern blots hybridized with the human ER cDNA. Steady-state ER protein levels for all three cell lines are shown in Fig. 4 as measured in a Western blot using the monoclonal antibody H222. Both mRNA and whole-cell extracts were prepared from the cells following 1, 3, 6, 12, 24, and 48 h exposure to either 1 nM E_2, 0.1 μM 4-OHT, or 0.1 μM ICI 182,780. As seen in Fig. 5, MCF-7:WS8 cells displayed a pattern of mRNA regulation consistent with previous studies (15, 18, 37). E_2 caused a dramatic decrease in the steady-state level of the ER mRNA in a time-dependent manner, whereas both antiestrogens had little effect on the steady-state mRNA levels. Exposure to E_2 as well as ICI 182,780 caused a dramatic reduction in the ER protein level. In contrast, the partial agonist 4-OHT caused an increase in the steady-state ER, reaching a peak at 24 h. This pattern is designated model I regulation.

Identical exposures of the T47D:A18 cell line gave rise to a distinctly different pattern of regulation (Fig. 6). Although the response of the ER mRNA and protein to 4-OHT was similar to that observed in the MCF-7:WS8 cells, the response to estradiol and ICI 182,780 was quite different. In the T47D:A18 cells, estradiol caused an initial decrease in the ER protein, as seen in the MCF-7:WS8 cells; however, this response was rapidly reversed, and by 12 h, the ER protein was present at control levels. The effect of E_2 on ER mRNA was also different than that observed in the MCF-7:WS8 cells, with a rapid increase in ER mRNA levels, which remained elevated for greater than 24 h. The ER protein response to ICI 182,780 exposure in the T47D:A18 cells was exactly as in the MCF-7:WS8 cells, with a nearly total loss of ER. However, the ER mRNA response in the T47D:A18 cells was quite different from that in the MCF-7:WS8 cells. The administration of ICI 182,780 to the T47D:A18 cells lead to an ~50% drop in ER mRNA by 6 h, and the ER mRNA remained repressed through the end of the experiment. We refer to this regulation as a model II response (see "Discussion").

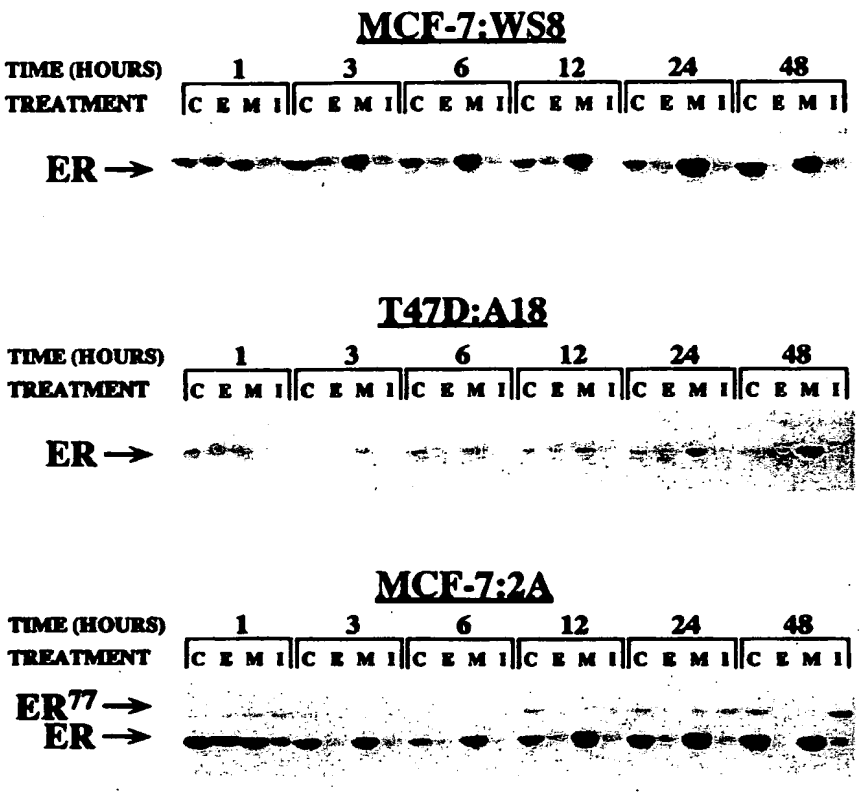


Fig. 4. Western blot analysis of steady-state ER levels in MCF-7:WS8, T47D:A18, and MCF-7:2A cell lines. Cells were treated as described in "Materials and Methods" with control (*C*), 1 nM E_2 (*E*), 1 μM 4-OHT (*M*), or 1 μM ICI (*I*) containing media for the indicated times. Whole-cell extracts were prepared, and equal amounts of protein were loaded into each lane. Blots were then probed using the monoclonal antibody H222 and visualized using enhanced chemiluminescence.

We also measured the effect of estrogens and antiestrogens on the ER in the estrogen-independent MCF-7:2A cell line (51). This cell line expresses two forms of the ER that can be easily differentiated in a Western blot. However, the mRNAs coding for these two respective receptors cannot be differentiated in a standard Northern blot. The duplication of exons 6 and 7 gives rise to an ~5% increase in size, and consequently the mRNAs coding for the two receptors cannot be resolved using this analysis. Therefore, changes in mRNA levels observed in the MCF-7:2A cell are a composite of the changes in both the wild-type and mutant ERs. Even with this limitation, the MCF-7:2A cells demonstrated a unique pattern of regulation of the two ERs (Fig. 7). Exposure to E_2 caused a repression of the ER mRNA and both the wild-type ER and the ER^{77}. The response to the antiestrogens was unique in the MCF-7:2A cells. The partial agonist 4-OHT caused an increase in the wild-type ER protein and a decrease in the ER^{77}. The effect of 4-OHT on the ER mRNA was also unusual, causing a decrease that was sustained for the length of the experiment. The most dramatic effect was observed in the differential effect of the pure antiestrogen ICI 182,780 on the protein level of the two ERs. The wild-type ER was rapidly decreased, as observed in the MCF-7:WS8 cells. In contrast, the ER^{77} protein level was increased following a short-lived decrease. The effect of ICI 182,780 on the ER mRNA was also unique, causing a rather dramatic increase in this message, which was continued throughout the length of the experiment.

DISCUSSION

We have described the regulation of ER mRNA and protein by estrogens and antiestrogens in three ER-positive breast cancer cell lines. These three cell lines exhibit a diversity of phenotypes, despite the fact that all express functional ER and induce PR synthesis as well as an estrogen-responsive reporter gene expression following estrogen exposure. Using these criteria, all three cells lines would be considered to be classically estrogen responsive. This classification would predict that these cells would respond to antiestrogens in a similar manner (57). Although growth of these cell lines is inhibited by both the partial agonist tamoxifen and the pure antiestrogen ICI 182,780, the studies described here demonstrate the diversity of ER regulatory mechanisms present in these cells.

We propose that the responses observed in these cell lines can be described with two models of ER regulation. MCF-7:WS8 cells exhibit a form of regulation that we refer to as model I. In this model, the primary response to an estrogenic stimulus is a feedback inhibition of steady-state ER mRNA levels. This model of regulation is also observed in other cultured cell lines, such as the breast cancer cell line ZR-75 (58) and the ovarian carcinoma line PEO4 (59). Another result of this model of regulation is an increase in ER following estrogen deprivation. This would allow a very rapid and vigorous response to estrogen stimulation following a period of estrogen deprivation. A model I response would be consistent with rapid cell proliferation, such as that observed in the uterus, where a few rounds of cell replication are necessary for each menstrual cycle. The estrogen- and progesterone-induced down-regulation of the ER would then attenuate this growth response and prevent uncontrolled proliferation. Previous studies have been reported that show this type of ER regulation in the uterus of experimental animals (13, 20, 22).

The second model, as observed in the T47D:A18 cells, is characterized by an increase of ER mRNA synthesis is response to estrogens. This model would require prolonged periods of estrogen exposure to induce maximal ER synthesis and response. This slower and more protracted response would be compatible with a differentiation response, such as the synthesis of vitellogenin in *Xenopus* and avian liver (60, 61).

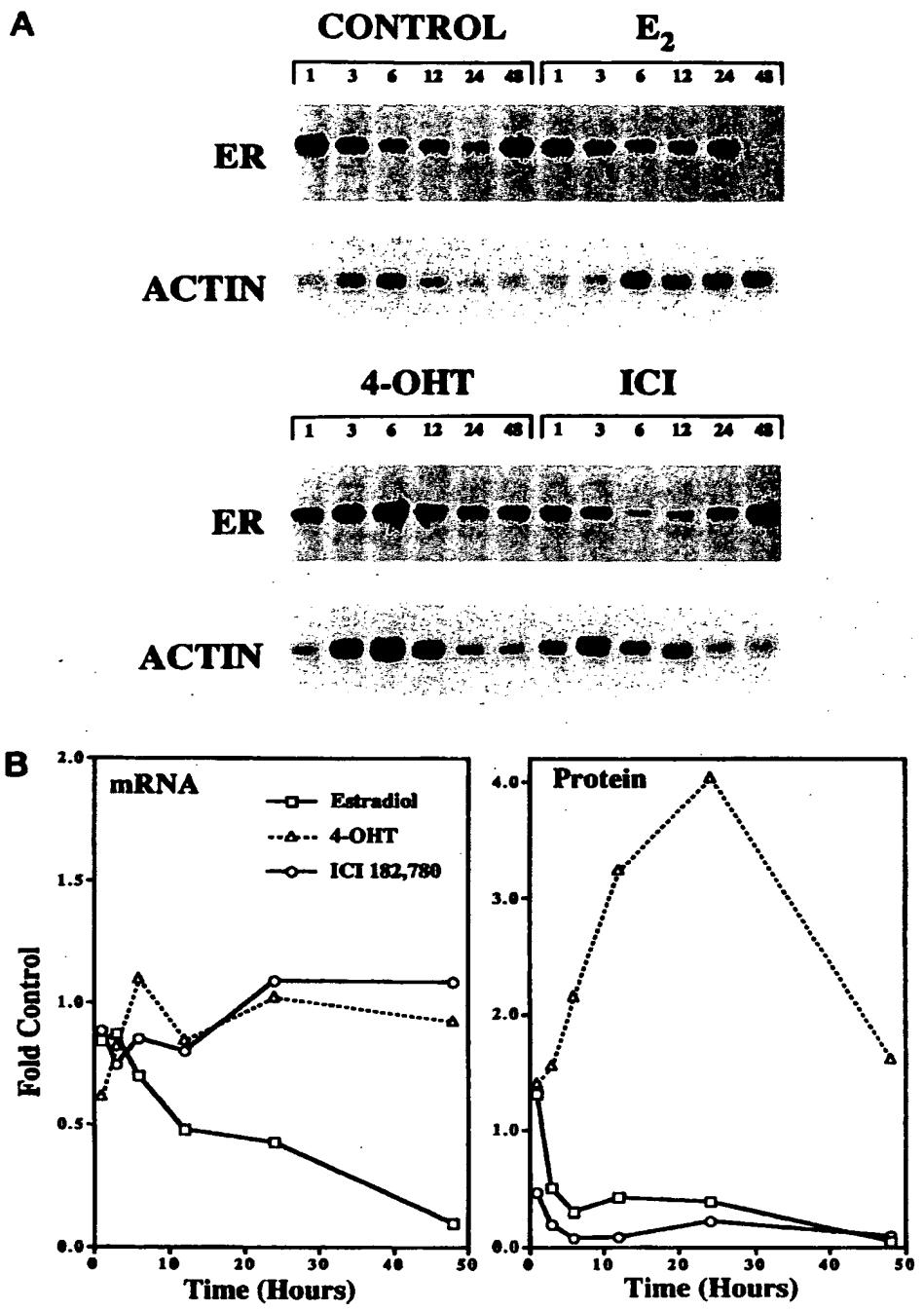


Fig. 5. Effects of E_2, 4-OHT, or ICI 182,780 on steady-state ER mRNA and protein levels in MCF-7:WS8 cells. Following estrogen deprivation for 4 days, cells were treated with control media, 1 nM E_2, 1 μM 4-OHT, or 1 μM ICI 182,780 (*ICI*) the next day, and protein and mRNA were harvested at the designated times as described in "Materials and Methods." *A*, representative Northern blot of MCF-7:WS8 poly(A)-enriched RNA probed with the human ER cDNA. To correct for loading and transfer differences, the blot was stripped and reprobed with β-actin. *B*, quantitation of relative ER protein and RNA levels following treatment with 1 nM E_2 (□), 1 μM 4-OHT (△), or 1 μM ICI 182,780 (○) for the indicated times.

The response observed in the MCF-7:2A cells is unique and serves to reinforce our finding in the MCF-7:WS8 cells. The M_r 66,000 ER responds in a model I fashion, with decreasing expression in the presence of estradiol and ICI 182,780 and increasing expression with 4-OHT. The ER^{77} is a unique marker of ER synthesis that does not bind ligand and, therefore, cannot be directly regulated by estrogens or antiestrogens. However, due to expression of the wild-type ER in these cells, any effects of ER on transcription and/or mRNA stability would still be functional. Therefore, all ligand-induced changes in ER^{77} protein level can be presumed to be the result of the wild-type ER effects on ER^{77} transcription or mRNA stability. This was confirmed in studies presented here that showed that the ER^{77} protein is not stabilized by 4-OHT binding or degraded by ICI 182,780 binding. The fact that ER^{77} is down-regulated by estradiol supports the hypothesis that the mechanism of model I regulation by estradiol is through repression of ER mRNA, at either a transcriptional or post-transcriptional level. We propose that the wild-type ER present in the MCF-7:2A cells can repress expression of both the wild-type ER and the ER^{77}. Furthermore, the increase in ER mRNA following ICI 182,780 exposure in the MCF-7:2A cells suggests that the ER^{77}

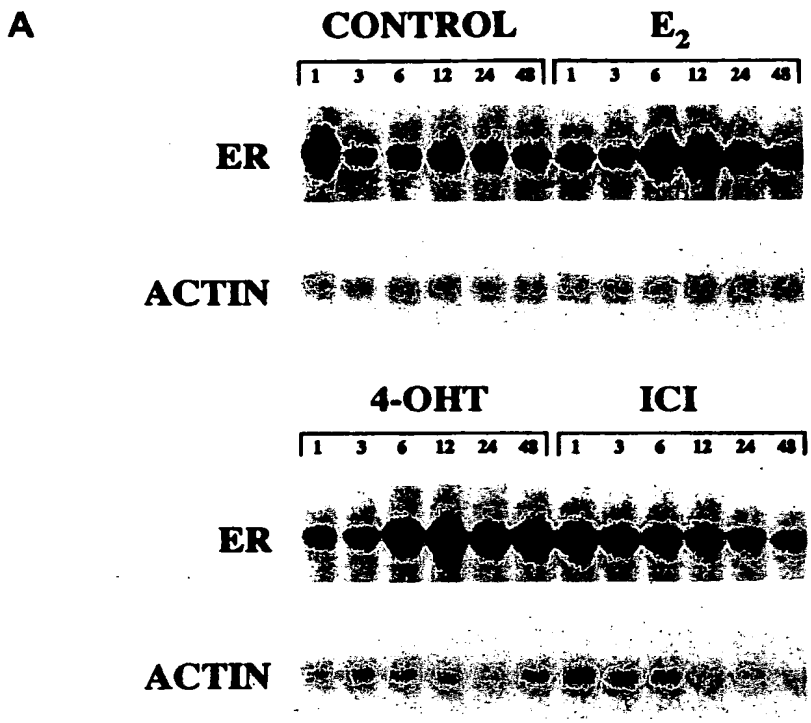
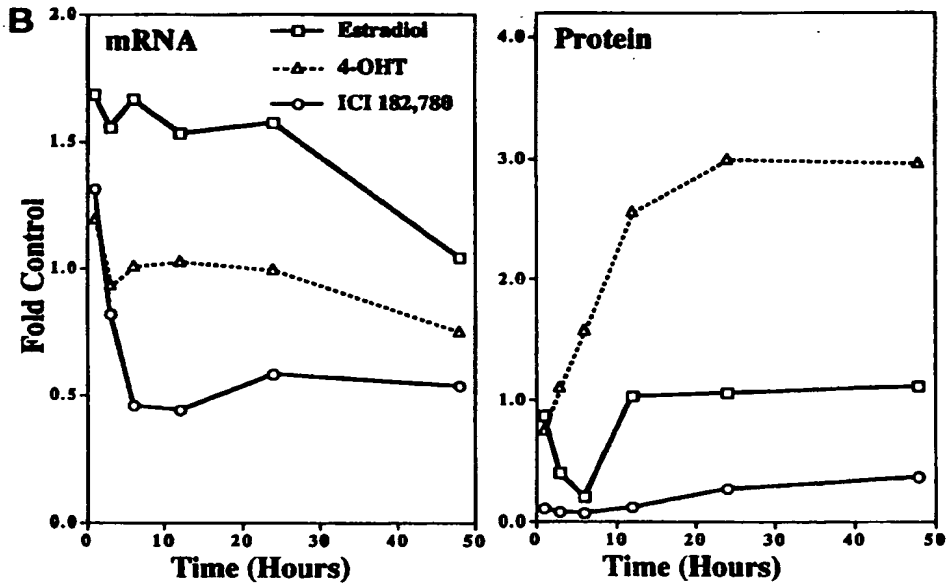


Fig. 6. Effects of E_2, 4-OHT, or ICI 182,780 on steady-state ER mRNA and protein levels in T47D:A18 cells. Following estrogen deprivation for 4 days, cells were treated with control media, 1 nM E_2, 1 μM 4-OHT, or 1 μM ICI 182,780 (*ICI*) the next day, and protein and mRNA were harvested at the designated times as described in "Materials and Methods." *A*, representative Northern blot of MCF-7:WS8 poly(A)-enriched RNA probed with the human ER cDNA. To correct for loading and transfer differences, the blot was stripped and reprobed with β-actin. *B*, quantitation of relative ER protein and RNA levels following treatment with 1 nM E_2 (□), 1 μM 4-OHT (△), or 1 μM ICI 182,780 (○) for the indicated times.



functions as an "unoccupied" receptor and causes a stimulation of ER transcription in a manner similar to that observed with estrogen withdrawal in model I regulation. This would argue that the complete loss of the ER following ICI 182,780 treatment is mechanistically different from estrogen withdrawal in these cells. It would also suggest that the transcription of the wild-type ER is being increased in the MCF-7:2A cells, but the increase in protein is not observed due to the rapid degradation of the ICI 182,780-occupied, wild-type ER. The concept of unoccupied ER exhibiting functions distinct from that in the complete lack of ER, as seen following ICI 182,780 exposure, is reinforced by the repression of ER mRNA in the T47D:A18 cells following ICI 182,780 treatment. If the loss of ER was no different than the presence of unoccupied ER, we would expect to see no change in the ER mRNA following ICI 182,780 exposure. In fact, ICI 182,780 causes a decrease in ER mRNA in the T47D cells.

Measurement of the DNA-binding ability of extracts isolated from MCF-7:WS8 and MCF-7:2A shows that the ICI 182,780-mediated effect on ER protein steady-state levels appears to require intact cells. Cells treated with ICI 182,780 for 24 h show a complete loss of ERE binding, whereas extracts isolated from control cells showed enhanced

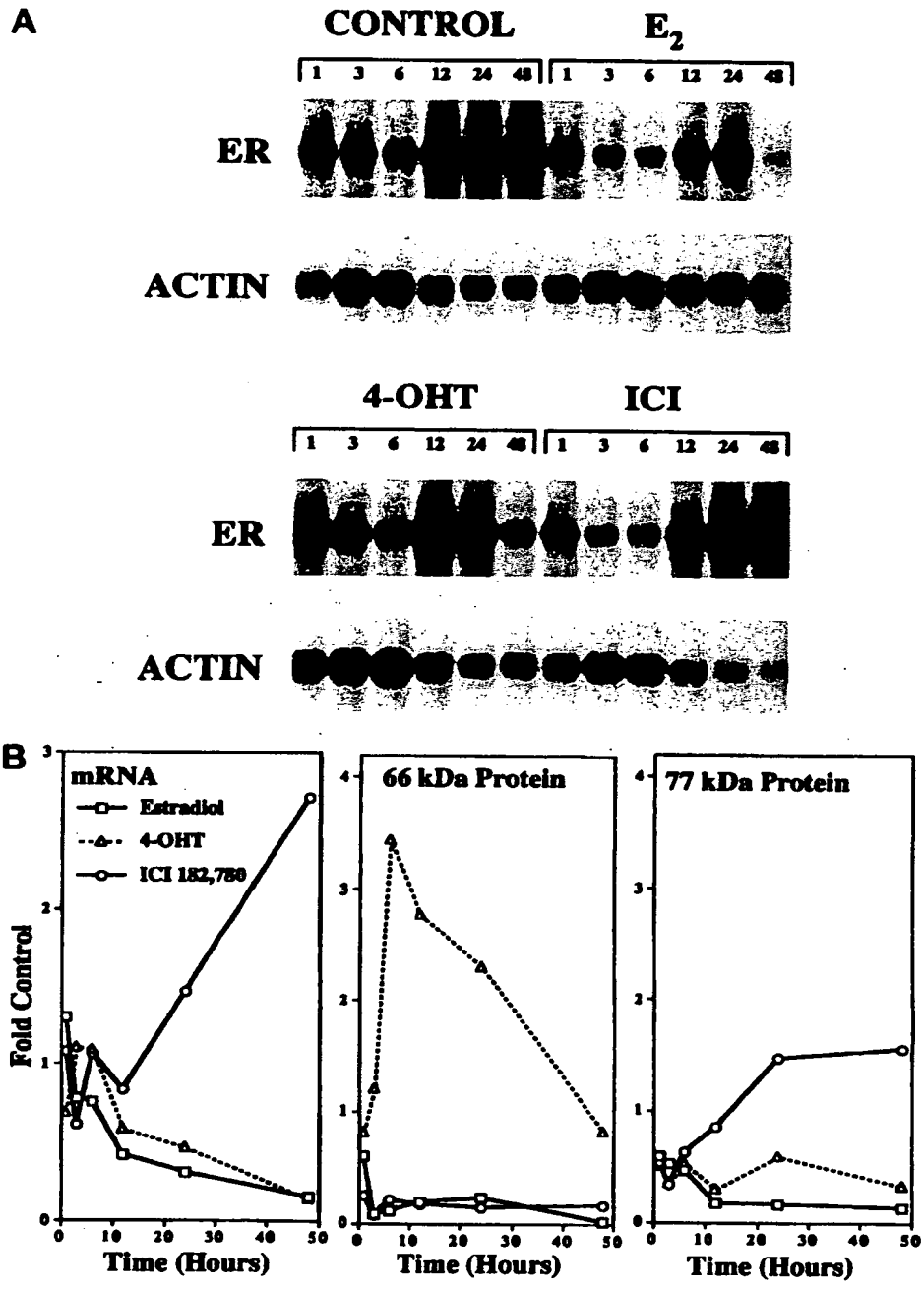


Fig. 7. Effects of E_2, 4-OHT, or ICI 182,780 on steady-state ER mRNA and protein levels in MCF-7:2A cells. Following estrogen deprivation for 4 days, cells were treated with control media, 1 nM E_2, 1 μM 4-OHT, or 1 μM ICI 182,780 (*ICI*) the next day, and protein and mRNA were harvested at the designated times as described in "Materials and Methods." *A*, representative Northern blot of MCF-7:WS8 poly(A)-enriched RNA probed with the human ER cDNA. To correct for loading and transfer differences the blot was stripped and reprobed with β-actin. *B*, quantitation of relative ER protein and RNA levels following treatment with 1 nM E_2 (□), 1 μM 4-OHT (△), or 1 μM ICI 182,780 (○) for the indicated times.

DNA binding when ICI 182,780 was added directly to the binding reactions. This also disproves previous reports that claimed that the pure antiestrogens function by inhibiting dimerization and, therefore, prevent DNA binding (62). These results suggest that the inhibition of growth and other ER-mediated responses following treatment with the pure antiestrogens is primarily the result of the rapid degradation of the ER (36–38).

The downstream effect of these two models of ER regulation is also reflected in the long-term effects of estrogen withdrawal on the MCF-7 and T47D cell lines. MCF-7 cell have for many years been shown to respond to short-term estrogen deprivation by up-regulation of the ER (33, 34). Our laboratory as well as others have isolated clones that have become estrogen independent following long-term (>8 months) estrogen deprivation. Interestingly, all estrogen-independent clones that have been characterized to date continued to express high levels of the ER (33, 34, 40, 63, 64). These clones also maintained sensitivity to the growth-inhibitory effects of antiestrogens. The response of the T47D cell line to long-term estrogen deprivation was quite different than that observed in the MCF-7:WS8 cells. We isolated estrogen-independent T47D clones that no longer express

measurable amounts of ER protein, as measured by Western blotting or specific E_2 binding.[5] A number of these initially ER-negative clones failed to express ER after more than 2 months in estrogen-containing media. Further investigation of two of these clones showed that they are completely insensitive to the effects of estrogens or antiestrogens. However, two other clones, which did not express ER protein when grown in estrogen-free media, were induced to express ER by growth for 2 months in estrogen-containing media. Continued growth in estrogen-containing media caused the development of a partially estrogen-responsive phenotype. This response is compatible with a model II response in which the ER expression is very low, in this case undetectable, but the addition of estrogens leads to a slow but steady increase in the ER expression, which eventually is observed as a measurable ER-mediated response.

The scarcity of ER-positive breast cancer cell lines forced us to focus our studies of ER effects on only a few unique cell lines. Although these cell lines proved very useful in the elucidation of many aspects of ER function, understanding of the vast diversity of potential mechanisms responsible for ER activity may not be represented in the models available to us. In the studies presented here, we demonstrated that the ER can be regulated in at least two distinct manners. We investigated the regulation of the ER in response to E_2 and two antiestrogens with distinct modes of action in two standard ER-positive breast cancer cell lines, as well as a unique cell line that has adapted to growth in estrogen-free media. The findings presented here suggest that ER regulation in breast cancer cells can be the result of either of two distinct models. Model I regulation is characterized by an ER that is expressed at high levels in the absence of estrogen and is subsequently down-regulated following estrogen binding, primarily through repression of the steady-state level of the mRNA. Model II regulation is distinguished by a low expression of the ER in an estrogen-free environment and the subsequent induction of ER expression following estrogen binding. The fact that two distinct forms of ER regulation are observed in these studies suggests that the regulation of the ER in the vast population of clinical breast cancers may show a variety of regulation mechanisms. Elucidation of these alternative responses to estrogens and antiestrogens is necessary for a complete understanding of growth control in breast cancer and the development of the next generation of therapeutic agents in the treatment of this disease.

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Antisense therapy targeting MDM2 oncogene in prostate cancer: Effects on proliferation, apoptosis, multiple gene expression, and chemotherapy

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This study was undertaken to investigate the role of mouse double minute 2 (MDM2) oncogene in prostate cancer growth and the potential of MDM2 as a target for prostate cancer therapy. An antisense anti-human–MDM2 mixed-backbone oligonucleotide was tested in human prostate cancer models with various p53 statuses, LNCaP ($p53^{wt/wt}$), DU145 ($p53^{mt/mt}$), and PC3 ($p53^{null}$). In a dose- and time-dependent manner, it specifically inhibited MDM2 expression and modified expression of several genes, at both mRNA and protein levels. In LNCaP cells, p53, p21, Bax, and hypophosphorylated retinoblastoma tumor suppressor protein (pRb) levels increased, whereas Bcl2, pRb protein, and E2F transcription factor 1 (E2F1) levels decreased. In DU145 cells, p21 levels were elevated and E2F1 levels decreased, although mutant p53, Rb, and Bax levels remained unchanged. In PC3 cells, MDM2 inhibition resulted in elevated p21, Bax, and pRb levels and decreased ppRb and E2F1 levels. In all three cell lines, MDM2 inhibition reduced cell proliferation, induced apoptosis, and potentiated the effects of the chemotherapeutic agents 10-hydroxycamptothecin and paclitaxel. The anti-MDM2 oligonucleotide showed antitumor activity and increased therapeutic effectiveness of paclitaxel in both LNCaP and PC3 xenografts, causing changes in gene expression similar to those seen *in vitro*. In summary, this study demonstrates that MDM2 has a role in prostate cancer growth via p53-dependent and p53-independent mechanisms and that multiple genes are involved in the process. MDM2 inhibitors such as second-generation antisense oligonucleotides have a broad spectrum of antitumor activities in human cancers regardless of p53 status, providing novel approaches to therapy of human prostate cancer.

p53 | p21 | Bax | E2F1 | oligonucleotides

Antisense therapy is designed to deliver to the target cells antisense molecules that target to mRNA with which they can hybridize and specifically inhibit the expression of pathogenic genes (1), thus offering the possibility of specific, rational, genetic-based therapy (1, 2). With encouraging results from preclinical and clinical studies in the past decade, significant progress has been made in the field (3–6). Although the first-generation phosphorothioate antisense oligonucleotides (oligo) are in clinical trials, a number of factors, including *in vivo* biostability and sequence motifs that could lead to unwanted effects, have been identified (4). The second-generation antisense oligos are designed to overcome these limitations (7, 8). Recently, we have developed several mixed-backbone antisense oligos (MBO) that are being tested for antitumor activity *in vitro* and *in vivo* (8–11).

Prostate cancer poses a major public health problem in the United States and worldwide. It has the highest incidence and is the second most common cause of cancer deaths in North American men, with estimates of 220,900 new cases and 28,900 deaths in 2003 (12). There is a need to develop novel therapeutic approaches. The molecular mechanisms of development and progression of prostate cancer are complicated and likely to involve multiple factors, such as tumor suppressor genes, oncogenes, growth factors, signal transduction molecules, adhesion molecules, and angiogenesis (13). Abnormal p53 expression occurs in prostate cancer patients (14–16). Mouse double minute 2 (MDM2) is amplified or overexpressed in a number of human tumors, including prostate cancer (17–21). The expression of MDM2 is induced by p53 and MDM2 oncoprotein functions as a negative regulator of p53. MDM2 also interacts with pRB, E2F transcription factor 1 (E2F1), and RNA, suggesting that MDM2 has p53-independent activities (21). Overexpression of MDM2 is associated with resistance to G_1/M growth arrest in prostate cancer DU145 cells ($p53^{mt/mt}$) (22) and with the androgen-independent phenotype (23), a characteristic of advanced prostate cancer. Therefore, we hypothesized that MDM2 may be a rational target for prostate cancer therapy.

Cancer chemotherapeutic agents and radiation often exert their cytotoxic effects through activation of wild-type p53, which may be limited in cancers with MDM2 expression. Therefore, inactivation of the MDM2–p53 negative feedback loop may increase the magnitude of p53 activation after DNA damaging treatments, thus enhancing therapeutic effectiveness. In addition, we further hypothesized that the role of MDM2 in tumor development and progression is not only associated with p53 pathway, but also with other p53-independent pathways. To that end, in the present study, we used *in vitro* and *in vivo* human prostate cancer models with distinct p53 genetic statuses, i.e., LNCaP (p53 wild type), DU145 (p53 mutant), and PC3 (p53 null), to demonstrate that the MDM2 antisense inhibitor has a broad spectrum of antitumor activity in human cancers regardless of p53 status. More importantly, in previous studies, we demonstrated the effect of MDM2 antisense oligo in human cancer cells with MDM2 overexpression (9); we now present data supporting the effect of second-generation antisense MDM2 inhibitor in cell lines that are not MDM2 overexpressors, implying that the application of the specific antisense anti-MDM2 oligo can be widened.

Materials and Methods

Antisense Oligonucleotides. Oligo AS, a 20-mer anti-human-MDM2 MBO (5′-UGACACCTGTTCTCACUCAC-3′) and its 5-base mismatch control, Oligo ASM (5′-UGTCACCCTTTT-TCATTUCAC-3′) were synthesized, purified, and analyzed as described (7, 10). Two nucleosides at the 5′ end and four nucleosides at the 3′ end are 2′-*O*-methylribonucleosides (represented by boldface letters); the remaining are deoxynucleosides. The underlined nucleosides of Oligo ASM are the sites of the mismatch controls compared with Oligo AS. All internucleotide linkages are phosphorothioate.

Abbreviations: MDM2, mouse double minute 2; oligo, oligonucleotide; MBO, mixed-backbone oligos; HCPT, 10-hydroxycamptothecin; Rb, retinoblastoma tumor suppressor protein; pRb, hypophosphorylated Rb; ppRb, hyperphosphorylated Rb; E2F1, E2F transcription factor 1.

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Cell Lines and Culture. The cell lines LNCaP, DU145, and PC3 were obtained from the American Type Tissue Culture Collection. LNCaP cells were cultured in RPMI medium 1640 supplemented with 4.5 g/liter glucose, 1% L-glutamine, 1.5 g/liter sodium bicarbonate, 1% Hepes buffer, and 1% sodium pyruvate. DU145 cells were cultured in EME medium (24). Media for PC3 cells consisted of Ham's F-12 medium supplemented with 1.5 g/liter sodium bicarbonate and 1% L-glutamine. All media contained 10% FBS and 1% penicillin/streptomycin. For *in vitro* experiments, cells were transfected with oligos in the presence of Lipofectin (7 μg/ml; Life Technologies, Gaithersburg, MD) and 1% FBS for various times before analysis of mRNA and protein levels, apoptosis, and cell proliferation. In combination treatments, oligo-transfected cells were incubated for an additional 12–36 h after adding chemotherapeutic agents, paclitaxel (Mead Johnson Oncology Products, Princeton, NJ) or 10-hydroxycamptothecin (HCPT) (Midwest, Beijing).

BrdUrd Cell Proliferation Assay. BrdUrd incorporation into cells was accomplished by using a BrdUrd cell proliferation assay kit from Oncogene Science. Cells were seeded in 96-well plates (8×10^3 to 1.2×10^4 cells per well) and transfected with oligos for 24 h (12 h for LNCaP). In combination treatments, cells were then exposed to paclitaxel for 36 h (12 h for LNCaP) or HCPT for 24 h (12 h for LNCaP). BrdUrd was added to the medium 10 h before treatment termination. The levels of BrdUrd incorporated into cells were quantified by anti-BrdUrd antibody, measuring absorbance at dual wavelengths of 450/540 nm with an OPTImax microplate reader (Molecular Devices, Sunnyvale, CA).

Detection of Apoptosis. Following a similar treatment protocol as above, cells in early and late stages of apoptosis were detected with an annexin V-FITC apoptosis detection kit from BioVision (Mountain View, CA) as described (24).

Western Blot Analysis. The protein levels of MDM2, p53, p21, hypophosphorylated retinoblastoma tumor suppressor protein (pRb), hyperphosphorylated pRb protein (ppRb), Bcl2, Bax, E2F1, and β-actin in cultured cells and xenografts were analyzed by using previously described methods, including densitometry measurement of protein bands (10, 11, 24). The monoclonal antibodies against MDM2 (Ab-1); p53 (Ab-6), p21 (Ab-1), Rb (Ab-5), Bcl2 (Ab-1), and Bax (Ab-2) were obtained from Oncogene Research Products (Boston, MA). Anti-E2F1 (KH95) monoclonal antibody was purchased from Santa Cruz Biotechnology. Anti-β-actin (SC-15) monoclonal antibody was obtained from Sigma.

Quantitation of mRNA. The mRNA levels in cells treated with Oligo AS or ASM were analyzed by RT-PCR. Cells were transfected with oligos as described above, and total RNA was extracted by using the Trizol reagent from Invitrogen, quantified by UV spectrophotometry, and used to create cDNA by using the SuperScript RT-PCR kit from Invitrogen. The PCR coamplifications of MDM2 (25), P53 (26), BAX (27), E2F1 (28), WAF1, RB, and BCL2 (29) with β-actin (29) were accomplished. The detailed methods are described in *Supporting Text* and Table 1, which are published as supporting information on the PNAS web site, www.pnas.org.

Xenograft Models. The models of LNCaP and PC3 xenografts were established by using the methods described previously (10, 11, 24). Male, 4- to 6-week-old severe combined immunodeficient mice (for LNCaP) and athymic nude mice (nu/nu) (for PC3), were obtained from Frederick Cancer Research and Development Center (Frederick, MD). Cultured cells were washed with and resuspended in serum-free media. The suspension (5×10^6 cells in 0.2 ml per mouse) mixed with Matrigel at a ratio of 1:1 (LNCaP) or 5:1 (PC3) was then injected into the left inguinal area of the mice. The mice were monitored by measuring tumor growth and body weight and by general clinical observation (24). Tumor-bearing mice were randomly divided into multiple treatment and control groups (five mice per group). All oligos, dissolved in physiological saline (0.9% NaCl), were given by i.p. injection at doses of 10 or 25 mg/kg per day, 5 days/week for 4 weeks. The control group received saline only. Paclitaxel (10 mg/kg) was given i.p., twice per week for 4 weeks. In combination therapy, oligos were given at 25 mg/kg level and paclitaxel as described above.

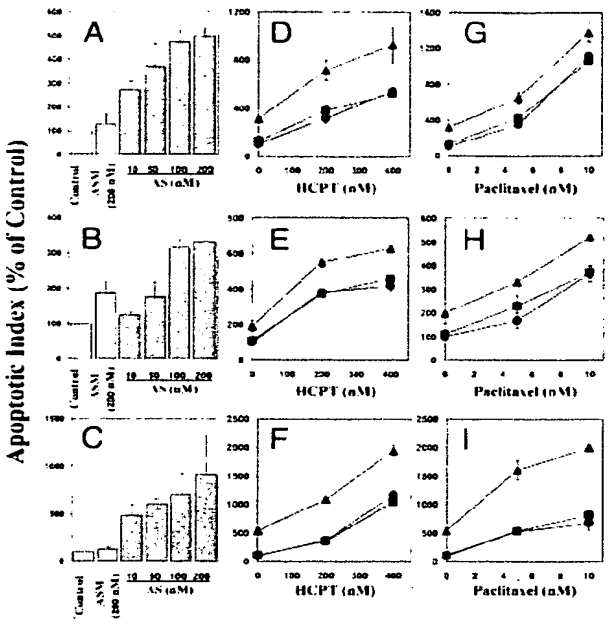

Fig. 1. Effects of Oligo AS alone or in combination with HCPT or paclitaxel on apoptosis in prostate cancer cells. The cells were transfected with Oligo AS or ASM at various concentrations for 12 h (*A*, LNCaP) or 24 h (*B*, DU145; *C*, PC3). In combination treatment with HCPT, the cells were transfected with 50 nM Oligo AS or ASM for 12 h (*D*, LNCaP) or 24 h (*E*, DU145; *F*, PC3) and then exposed to HCPT for an additional 12 h (*D* and *E*) or 24 h (*F*). In combination treatment with paclitaxel, the cells were transfected with 50 nM Oligo AS or ASM for 12 h (*G*, LNCaP) or 24 h (*H*, DU145; *I*, PC3) and then exposed to paclitaxel for an additional 12 h (*G*) or 36 h (*H* and *I*). Cells that stained positive for annexin V-FITC (early apoptosis) and positive for FITC and propidium iodide (late apoptosis) were counted. Relative levels of apoptotic indices were expressed as percentage of Lipofectin control. (*D–I*) Filled circles, Lipofectin control; filled squares, Oligo ASM 50 nM; filled triangles, Oligo AS 50 nM.

Results

In Vitro* MDM2 Antisense Inhibition Resulted in Induction of Apoptosis, Inhibition of Cell Proliferation, and Chemosensitization in Prostate Cancer Cells, Regardless of p53 Status.** ***Induction of apoptosis. In a dose-dependent manner, Oligo AS induced apoptosis in all three prostate cancer cell lines, regardless of p53 status (Fig. 1). After treatment with 200 nM Oligo AS, the apoptotic index increased by 400% in LNCaP cells (Fig. 1*A*), 230% in DU145 cells (Fig. 1*B*), and 820% in PC3 cells (Fig. 1*C*). The mismatch control Oligo ASM had no or minimal effects. Pretreatment with 50 nM Oligo AS, but not the control Oligo ASM, sensitized all of the three cell lines to the cancer chemotherapeutic agents HCPT (Fig. 1 *D–F*) and paclitaxel (Fig. 2 *G–I*).

Inhibition of cell proliferation. In a dose-dependent manner, Oligo AS inhibited proliferation, as measured by BrdUrd incorporation, in all three prostate cancer cell lines, regardless of p53

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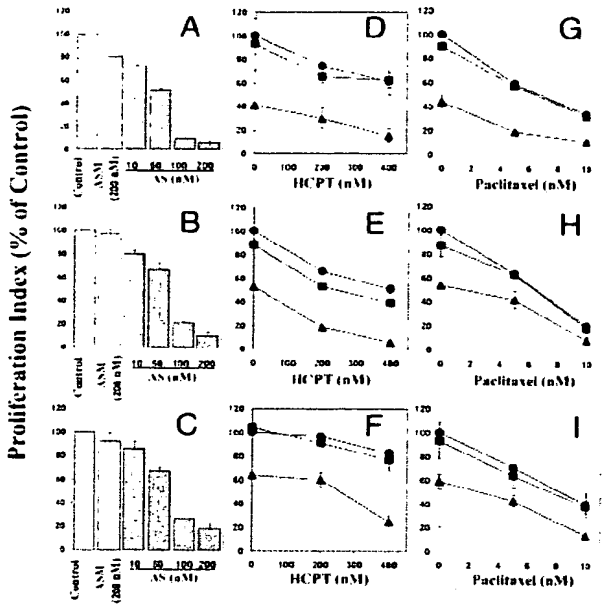

Fig. 2. Effects of Oligo AS on the proliferation of prostate cancer cells, analyzed by BrdUrd incorporation. By using the same treatment protocol as for Fig. 1, LNCaP (*A*, *D*, and *G*), DU145 (*B*, *E*, and *H*), and PC3 (*C*, *F*, and *I*) cells were treated with oligos alone (*A–C*) or in combination with HCPT (*D–F*) or paclitaxel (*G–I*). Relative levels of BrdUrd incorporations were expressed as percentage of Lipofectin control. (*D–I*) Filled circles, Lipofectin control; filled squares, Oligo ASM 50 nM; filled triangles, Oligo AS 50 nM.

status (Fig. 2). After treatment with 200 nM Oligo AS, the proliferation index decreased by 94% in LNCaP cells (Fig. 2*A*), 90% in DU145 cells (Fig. 2*B*), and 82% in PC3 cells (Fig. 2*C*), respectively. The mismatch control Oligo ASM had no or minimal effects. Pretreatment with 50 nM of Oligo AS, but not ASM, sensitized all of the three cell lines to HCPT (Fig. 2 *D–F*) and paclitaxel (Fig. 2 *G–I*).

In Vitro* MDM2 Antisense Inhibition Modified Multiple Gene Expression.** ***LNCaP cells. Both MDM2 protein and mRNA were inhibited by Oligo AS in a dose-, time-, and sequence-dependent manner (Fig. 3). The MDM2 knockdown resulted in p53 protein elevation in a dose- and time-dependent fashion (Fig. 3 *A* and *B*). However, P53 mRNA levels did not change (Fig. 3 *C* and *D*), confirming that inactivation of p53 by MDM2 is mainly via the p53 degradation pathway. The levels of p21 and Bax proteins and mRNAs were also elevated. There were also increases in pRb and RB mRNA and decreases in ppRb. Both E2F1 protein and mRNA levels decreased. The Bcl2 protein level decreased (Fig. 3 *A* and *B*), but its mRNA level was not changed (Fig. 3 *C* and *D*).

DU145 cells. In a dose-, time-, and sequence-dependent manner, MDM2 protein and mRNA levels were inhibited by Oligo AS (Fig. 4). No changes in mutant p53 protein and mRNA were observed. Interestingly, both p21 protein and mRNA levels were elevated, which is independent of p53. E2F1 protein levels were decreased (Fig. 4 *A* and *B*) without remarkable changes in mRNA levels (Fig. 4 *C* and *D*). DU145 cells are Bcl2 null and express mutant Bax and Rb, which were not changed after MDM2 inhibition.

PC3 cells. After antisense MDM2 inhibition, p21 mRNA and protein levels were significantly elevated (Fig. 5). Bax protein levels were also elevated (Fig. 5 *A* and *B*) without remarkable changes in mRNA levels (Fig. 5 *C* and *D*). The pRb protein levels

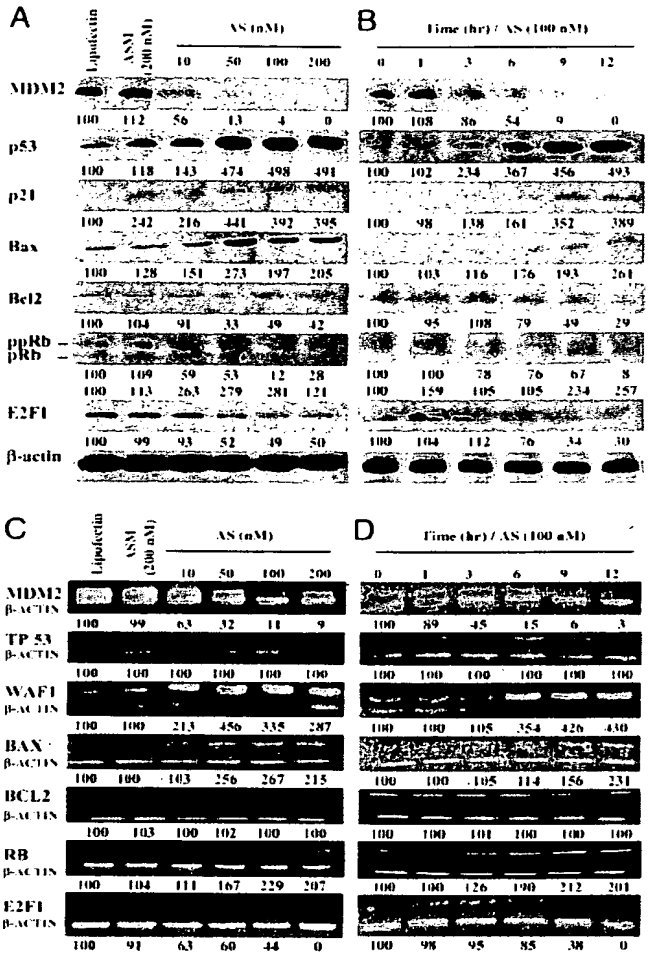

Fig. 3. Protein (*A* and *B*) and mRNA (*C* and *D*) levels of various genes in LNCaP cells treated with Oligo AS or controls. (*A*) The cells were transfected with Oligo AS (10–200 nM) or ASM (200 nM) for 24 h. Proteins were analyzed by Western blotting. The number under each band is expressed as a percentage of Lipofectin control, normalized by the corresponding β-actin level. (*B*) Cells were incubated with Oligo AS (100 nM) for various times. (*C*) Cells were transfected as in *A*. Target mRNAs were analyzed by RT-PCR and coamplified with β-actin mRNA. The number under each band is expressed as a percentage of Lipofectin control, normalized by the corresponding coamplified β-actin level. (*D*) Cells were incubated with Oligo AS (100 nM) for various times.

increased and ppRb levels decreased (Fig. 5 *A* and *B*) without significant changes in RB mRNA levels (Fig. 5 *C* and *D*). E2F1 protein levels decreased (Fig. 5 *A* and *B*) without changes in its mRNA levels (Fig. 5 *C* and *D*). No changes in Bcl2 protein or mRNA levels were found (Fig. 5).

In Vivo* MDM2 Antisense Inhibition Showed Dose-Dependent Antitumor Activity and Chemosensitization and Modified Multiple Gene Expression in Prostate Cancer Xenografts.** ***LNCaP xenografts. Oligo AS showed significant antitumor activity in a dose-dependent manner (Fig. 6*A*) and increased therapeutic effectiveness of paclitaxel in severe combined immunodeficient mice bearing LNCaP xenografts (Fig. 6*B*). Oligo ASM showed minimal effects on tumor growth or on paclitaxel efficacy, demonstrating the specificity of Oligo AS (Fig. 6 *A* and *B*). To confirm the antisense mechanism *in vivo*, a separate study was accomplished to analyze protein levels of MDM2 and related genes, by Western blot

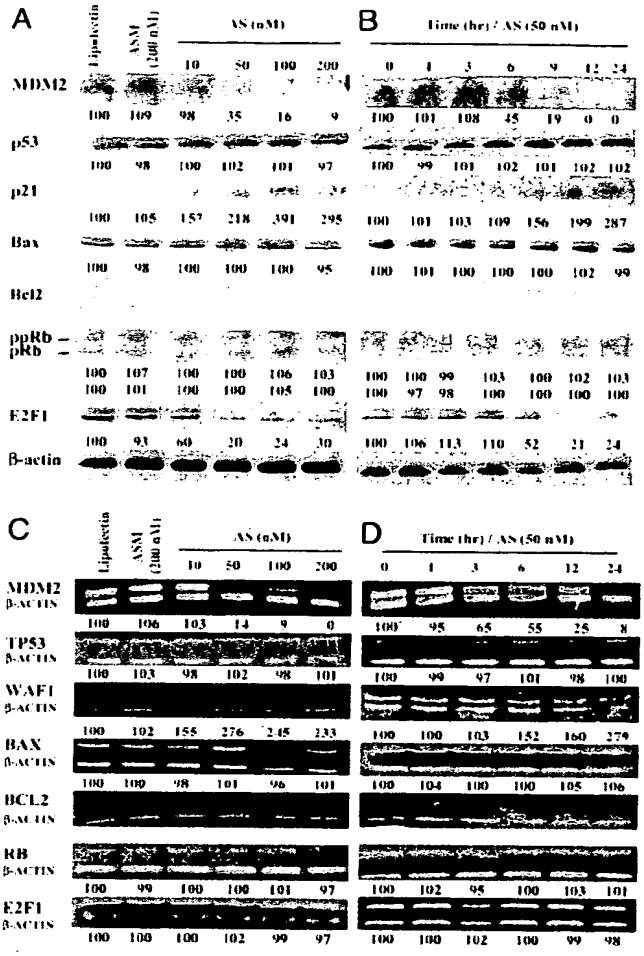

Fig. 4. Protein (*A* and *B*) and mRNA (*C* and *D*) levels of various genes in DU145 cells treated with Oligo AS or controls. The assay conditions were the same conditions as described for Fig. 3, except for the concentration of Oligo AS used in *B* and *D* (50 nM).

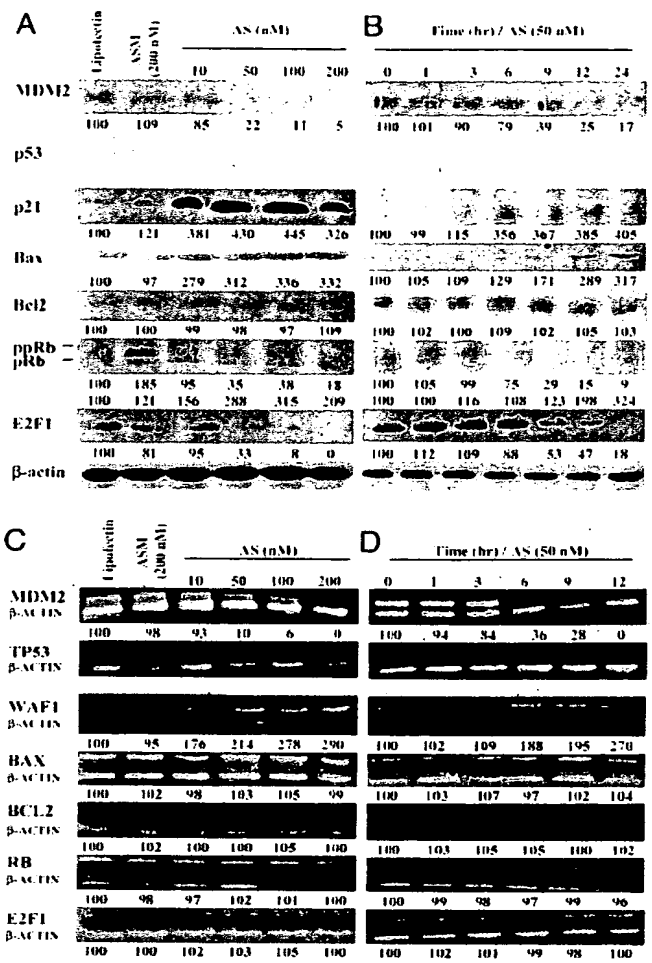

Fig. 5. Protein (*A* and *B*) and mRNA (*C* and *D*) levels of various genes in PC3 cells treated with Oligo AS or controls. The assay conditions were the same conditions as described for Fig. 3, except for the concentration of Oligo AS used in *B* and *D* (50 nM).

analysis of pooled LNCaP xenografts. As shown in Fig. 6*C*, Oligo AS specifically inhibited MDM2 expression in a dose-dependent manner, resulting in elevation of p53, p21, Bax, and pRb and reduction of ppRb, Bcl2, and E2F1. The control Oligo ASM had no or minimal effects.

PC3 xenograft model. The antitumor activity of Oligo AS was further shown in the PC3 model, which is p53 null, providing direct evidence for p53-independent activity of MDM2 (Fig. 7*A*). In this model, Oligo AS also increased therapeutic effectiveness of paclitaxel (Fig. 7*B*). Oligo ASM showed no effect (Fig. 7 *A* and *B*). The protein levels of MDM2 were specifically decreased by Oligo AS in a dose-dependent manner, resulting in elevation of p21, Bax, and pRb and reduction of ppRb and E2F1 (Fig. 7*C*). Further quantitative analysis of the mode of chemosensitization effects in both LNCaP and PC3 models is presented in *Supporting Text* and Table 2, which is published as supporting information on the PNAS web site.

Discussion

Antisense oligonucleotides have been extensively studied as a research tool in determining gene function and as a novel therapeutic approach to treatment of various human diseases (1–6). However, there still are many questions regarding their specificity and efficacy. For the first-generation antisense oligos, there are several nonspecific effects that detract from their clinical efficacy and safety profiles (3, 4). In the present study, we used various *in vitro* and *in vivo* models to determine the specific antitumor activity and the underlying mechanism of action of the anti-human-MDM2 MBO, a new generation of antisense oligos. Unlike most reported sequences of first-generation antisense oligos under clinical investigation (4), our test and control oligos do not contain any CpG motifs or other sequences known to have nonspecific, non-antisense effects.

In the present study, we have demonstrated at least six noteworthy results. First, the novel anti-MDM2 MBO, Oligo AS, specifically knocked-down MDM2 expression, as demonstrated at both mRNA and protein levels, in a dose- and time-dependent manner, regardless of p53 status. Second, MDM2 inhibition resulted in enhanced apoptosis and decreased cell proliferation in a dose-dependent, sequence-specific manner, regardless of p53 status. Third, in a dose-dependent manner, Oligo AS demonstrated sequence-specific *in vivo* antitumor activity in both LNCaP ($p53^{wt/wt}$) and PC3 ($p53^{null}$) xenografts. Fourth, MDM2 inhibition resulted in chemosensitization *in vitro* and *in vivo*, regardless of p53 status. Fifth, in a dose- and time-

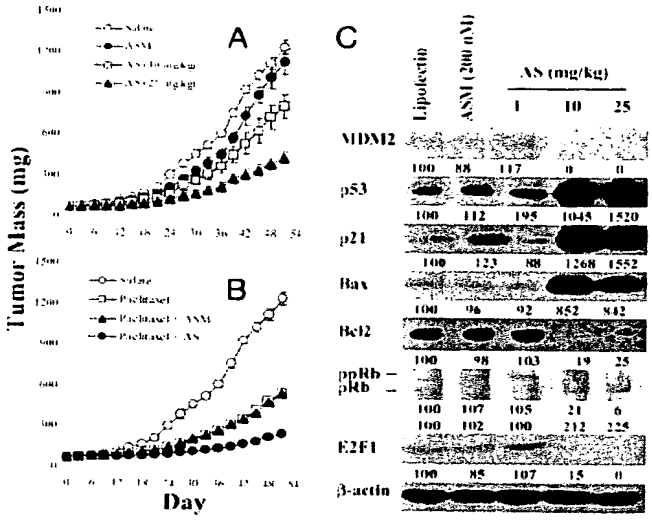

Fig. 6. *In vivo* antitumor activity of Oligo AS administered alone (*A*) or in combination with paclitaxel (*B*) in severe combined immunodeficient (SCID) mice bearing LNCaP xenografts and the protein expression profile of xenografts (*C*). (*A*) Oligo AS (10 or 25 mg/kg per day) or ASM (25 mg/kg per day) treatments were initiated on day 0 and given i.p., 5 days/week for 4 weeks. (*B*) Oligos were given i.p. at 25 mg/kg per day, 5 days/week for 4 weeks, and paclitaxel was given i.p. at 10 mg/kg per day, twice per week for 4 weeks. (*C*) The SCID mice bearing LNCaP xenografts (60–70 mg) were treated i.p. with Oligo AS (10 or 25 mg/kg) or ASM (25 mg/kg) for 7 consecutive days. Proteins from tumor homogenates were analyzed by Western blotting. The number under each band is expressed as a percentage of saline control, normalized by the corresponding β-actin level.

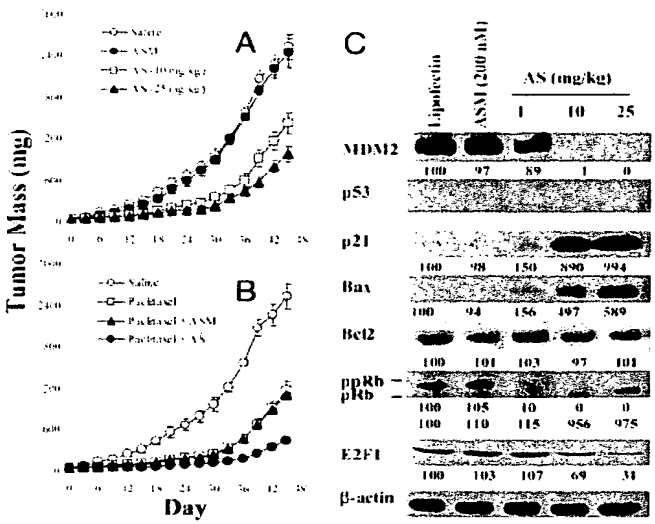

Fig. 7. *In vivo* antitumor activity of Oligo AS administered alone (*A*) or in combination with paclitaxel (*B*) in nude mice bearing PC3 xenografts and the protein expression profile (*C*). The treatment and analytical procedures were the same as described for Fig. 6.

dependent manner, Oligo AS modified the expression of several genes, with varying profiles in cells with different p53 status. In the LNCaP cells ($p53^{wt/wt}$), MDM2 inhibition resulted in significant elevation of the protein levels of p53, p21, pRb, and Bax and reduction of ppRb, Bcl2, and E2F1. The changes in mRNA levels did not always correspond to those in protein levels, suggesting that there are multiple mechanisms of action for MDM2 inhibition. In DU145 cells ($p53^{mt/mt}$), MDM2 inhibition also resulted in elevation of p21 at both mRNA and protein levels, and decreases in E2F1 protein levels. In PC3 cells, significant elevation of the protein levels of p21, Bax, and pRb and reduction of ppRb and E2F1 were observed. Finally, *in vivo* dose-dependent modulation of expression in several genes after MDM2 inhibition was demonstrated in both LNCaP and PC3 xenograft models, consistent with the above *in vitro* findings. Our results provide direct evidence supporting sequence-specific *in vitro* and *in vivo* antisense efficacy of the anti-MDM2 oligo, which should be considered as a proof of principle of antisense technology.

Thus far, most investigations of MDM2 functions focus on its interaction with p53. As a negative regulator of p53, MDM2 plays an important role in tumor formation and growth. In tumors with wild-type p53 expression, MDM2 interacts with p53 via various mechanisms such as inhibition of its transactivation activity (30) and facilitation of its degradation (21). Therefore, inhibition of MDM2 is a promising means of restoring p53 function, including p21 and Bax induction, resulting in cell growth arrest and/or apoptosis. Our results with the *in vitro* and *in vivo* LNCaP models provide direct evidence for p53–MDM2 interaction.

The tumorigenic properties of MDM2, however, may be independent of p53 (21). Approximately one-third to one-half of human prostate cancers have MDM2 overexpression with or without p53 expression (20, 21), suggesting that MDM2 may be involved directly in the development of prostate cancer. Moreover, the induction of MDM2 may be associated with resistance to chemotherapy and radiation therapy in prostate cancer patients (31, 32). Our results with the DU145 and PC3 models suggest a p53-independent activity of MDM2, which may have therapeutic applications, because the majority of advanced prostate cancers contain no p53 expression or mutant p53 (13–20) and because MDM2 is associated with the androgen-independent phenotype (23).

Unlike other published reports, the present study demonstrated changes in expression of multiple genes, not only the targeted gene, an observation that is important to understanding the mechanism of action for antisense therapy. Furthermore, the expression profiles were analyzed at both protein and mRNA levels. After MDM2 inhibition, the expression of p21 and Bax was observed *in vitro* and *in vivo*, regardless of p53 status. Although WAF1 (p21) gene is a reporter gene of p53 activity, several reports demonstrate that p53-independent p21 induction is mediated by various stimuli, including epidermal growth factor and fibroblast growth factor (20), silibinin (31), zinc (33), and genistein (34). The lack of p21 expression is correlated with poorer clinical outcome in bladder, colon, and hepatocellular carcinomas, and down-regulation of p21 is involved in the development of androgen-independence in prostate cancer (20). Our results from the present study support the concepts that induction of p21 is both p53-dependent and p53-independent and that MDM2 inhibition affects both p21 protein and mRNA levels.

The expression of Bax may also be controlled by p53 (35) or p53-independent mechanisms such as proteosome inhibition (36). In the present study, we demonstrated Bax induction in both LNCaP ($p53^{wt/wt}$) and PC3 ($p53^{null}$) cell lines, after MDM2 inhibition. Because Bax degradation by proteosome may be an important regulation mechanism in advanced prostate cancer (36) and because MDM2 has ubiquitin ligase activity (21), MDM2 may have a direct role in Bax regulation. The ratio of Bcl2/Bax is important in determining which cells undergo apoptosis and which survive after DNA damage (37). Bcl2 up-regulation is associated with androgen independence and

resistance to chemotherapy (38). In the present study, we report that MDM2 inhibition results in simultaneous Bcl2 reduction and Bax elevation in LNCaP cells and xenografts, explaining partly the significant antitumor and chemosensitization effects of the antisense therapy.

The RB tumor suppressor is involved in cell cycle control and differentiation by modulating the activity of transcription factors such as E2F protein family (39); its mutations or deletions are evident in human prostate cancer (40). ppRb promotes cell progression (39). pRb regulates the G_2/M transition (41), inhibiting androgen-independent development (42) and inducing p53-dependent (43) or p53-independent apoptosis (41). The elevated p21 after MDM2 inhibition may lead to Rb hypophosphorylation. However, the RB mRNA increase in LNCaP cells may be p53-dependent, because no changes in RB mRNA were observed in PC3 ($p53^{null}$) cells.

E2F1 stimulates cellular proliferation and cell cycle progression from G_1 to S phase (44–46) and correlates with increased tumor cell invasiveness and metastasis (45). In addition to being negatively regulated by pRb, it was activated by MDM2 directly (21). Results from the present study further demonstrate that MDM2 inhibition results in decrease of E2F1, regardless of p53 status, indicating a role for E2F1 in the antitumor activity and chemosensitization effects of the anti-MDM2 oligo.

In summary, although the role of MDM2 in the development and progression of human cancer was originally thought to be associated with p53 inactivation, we conclude, based on our results from *in vitro* and *in vivo* MDM2 antisense treatment in various prostate cancer models, that MDM2 has both p53-dependent and p53-independent activities. MDM2 inhibitors, such as second-generation antisense oligos described in this report, may have a broad spectrum of antitumor activity in human cancers, regardless of p53 status, providing a basis for future development of this novel approach to the therapy of human cancers.

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Constitutive Activation of Stat3 in Human Prostate Tumors and Cell Lines: Direct Inhibition of Stat3 Signaling Induces Apoptosis of Prostate Cancer Cells[1]

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ABSTRACT

Signal transducers and activators of transcription (STATs) were identified originally as key components of cytokine signaling pathways. More recently, constitutive activation of STAT proteins has been detected in a wide variety of human tumor specimens and tumor cell lines. Here, we examined the activation of one STAT family member, Stat3, in human prostate cancer cell lines and primary prostate tumors. An analysis of 45 adenocarcinomas obtained at radical prostatectomy revealed elevated levels of constitutive Stat3 activation in 37 (82%) of 45 of the tumors compared with matched adjacent nontumor prostate tissues. A highly specific immunohistochemical assay for detection of phospho-Stat3 revealed that elevated Stat3 activity was localized primarily in the tumor cells of prostate carcinoma specimens. Furthermore, higher levels of Stat3 activation in patient specimens were correlated significantly with more malignant tumors exhibiting higher Gleason scores. In addition, all of the three human prostate cancer cell lines examined (DU145, PC3, and LNCaP) displayed constitutive activation of Stat3. Substantially lower levels of Stat3 activation were detected in LNCaP cells; however, stimulation with interleukin 6 induced a significant increase in Stat3 DNA-binding activity in these cells. Moreover, the direct inhibition of constitutive Stat3 signaling in DU145 cells using antisense Stat3 oligonucleotides induced growth inhibition and apoptosis. Our findings demonstrate that constitutive activation of Stat3 occurs frequently in primary prostate adenocarcinomas and is critical for the growth and survival of prostate cancer cells. These studies further suggest that Stat3 signaling represents a potentially novel molecular target for prostate cancer therapy.

INTRODUCTION

The natural history of prostate cancer is poorly understood, but progression appears to be related to stage and grade of tumor (reviewed in Refs. 1–4). Biologically, prostate cancer represents a heterogeneous disease entity that exhibits various degrees of aggressiveness, patterns of metastasis, and response to therapy. Therefore, there is a great need to identify molecular prognostic factors that allow stratification of prostate cancer patients into homogeneous treatment groups (4, 5). Furthermore, the identification of new molecular targets may lead to more effective treatments for prostate cancer.

STATs[4] were identified originally as central players in signaling pathways involved in mediating responses to IFNs and other cytokines (reviewed in Refs. 6–8). On phosphorylation of STAT monomers by tyrosine kinases, the monomers dimerize, translocate to the nucleus, and bind to specific promoter sequences, thereby inducing gene expression. STAT proteins have essential functions in signaling pathways critical to normal cellular processes including immune function, development, differentiation, proliferation, and survival (9–12). One of the cytokines known to activate Stat3 is IL-6. IL-6 regulates a wide variety of biological responses and stimulates or inhibits cellular growth in a cell type-dependent manner (13, 14). Furthermore, activation of Stat3 by IL-6 has a critical role in the pathogenesis of multiple myeloma by preventing apoptosis (15). In addition, IL-6 has been suggested to participate in malignant progression of prostate cancer (16).

Earlier studies have reported that constitutive activation of one STAT family member, Stat3, occurs in fibroblasts stably transformed by the Src oncoprotein and other oncoproteins that activate tyrosine kinase signaling pathways (17–20). More recent studies have demonstrated that Stat3-mediated expression of cellular genes is required for Src-induced oncogenic transformation (21–23). In addition, constitutive STAT activation occurs frequently in a variety of human tumor cell lines and primary human tumors including leukemias, lymphomas, multiple myeloma, head and neck cancer, and breast cancer (24–27). Moreover, a constitutively activated mutant of Stat3 is sufficient to induce some properties of transformed cells, including tumorigenicity (28). Collectively, these findings provide evidence that signaling by Stat3 protein participates in regulating the processes of cell growth and survival during oncogenesis.

Because cytokines including IL-6 have been implicated in prostate cancer, we examined Stat3 signaling in primary tumor specimens from prostate cancer patients and in human prostate cancer cell lines. Using both a biochemical Stat3 DNA-binding assay and an immunohistochemical phospho-Stat3 assay, we demonstrate that Stat3 is constitutively activated with high frequency in prostate adenocarcinomas compared with matched adjacent nontumor prostate tissues. In addition, we used antisense Stat3 oligonucleotides to block Stat3 signaling and to evaluate the biological role of activated Stat3 in model prostate cancer cell lines. Results indicate that activated Stat3 transduces signals that are required for growth and survival of human prostate cancer cells. Our findings implicate constitutive activation of Stat3 in malignant progression of prostate cancer and suggest that Stat3 represents a promising molecular target for novel prostate cancer therapy.

MATERIALS AND METHODS

Patient Identification. In contrast to other solid tumors, prostate cancer is not easily identified by gross inspection. Because of the wide use of PSA screening and early diagnosis, a significant number of patients who undergo radical prostatectomy reveal very little residual tumor. To assure that enough tumoral tissue was collected from the prostatectomy specimens to perform all of the proposed molecular studies, we used only prostatectomy specimens from patients with a prior diagnosis of prostate cancer in at least 50% of two of six prostate biopsies. The ideal patient was the one with two or three positive biopsies in one side and negative biopsies on the contralateral side. The findings on the prostate biopsies, which are routinely performed before pros-

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[4] The abbreviations used are: STAT, signal transducer(s) and activator(s) of transcription; IL, interleukin; FBS, fetal bovine serum; EMSA, electrophoretic mobility shift assay; hSIE, high affinity variant of the sis-inducible element.

latectomy, were used for patient selection and as guidance for tumoral and nontumoral tissue collection.

Pathology Evaluation. Prostatectomy specimens were sent immediately to the pathology laboratory on ice with a label indicating the time of excision (to monitor processing time from excision to snap-freezing). Every effort was made to minimize processing time to less than 15 min because it was extremely important to snap-freeze tumor specimens in liquid nitrogen as soon as possible to preserve the activation state of STAT proteins in tumor cells. The specimen was cut at 5-mm intervals, and areas of normal and tumor tissue were identified grossly with the guidance of biopsy results obtained before prostatectomy. The frozen sections were evaluated by the pathologists (J. D., L. B. M.) to confirm the diagnosis of both normal and tumor tissue. This approach assured reliable comparison of biochemical data in tumoral and nontumoral tissue. Excess tissue not required for pathological examination of the specimen was obtained for the purposes of this study. Informed consents were signed by all patients to allow the use of their tissues in these experiments as part of a clinical protocol approved by the local Institutional Review Board. Tissue adjacent to (mirror-image area) the confirmed tumor or normal tissue by frozen section evaluation was snap-frozen immediately in liquid nitrogen and sent to the Moffitt Cancer Center Tumor Bank for future STAT studies. The remainder of the prostatectomy specimen was fixed in formalin and processed for routine histological examination and immunohistochemical analysis.

Cell Lines and Culture Conditions. The human prostate carcinoma cell lines LNCaP, DU145, and PC3 were obtained from the American Type Culture Collection (Rockville, MD) and were grown in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FBS. IL-6 stimulation experiments were performed using 10 ng/ml IL-6 in serum-free medium for the indicated times.

Nuclear Extract Preparation and EMSA. For STAT analysis by EMSA, the frozen tissue specimens were placed in a prechilled mortar and pestle on liquid nitrogen, and the tissue was pulverized in the frozen state. Nuclear extracts were prepared as described previously (17, 20) from primary tissue specimens with approximately 10^6 cells by high-salt extraction into 30–70 μl buffer [20 mM HEPES (pH 7.9), 420 mM NaCl, 1 mM EDTA, 20% glycerol, 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 μM aprotinin, 1 μM leupeptin, and 1 μM antipain] producing about 30–90 μg of total protein. For EMSA, 5 μg of total extracted protein was used for each lane. EMSA was performed using a ^{32}P-radiolabeled oligonucleotide probe containing a hSIE derived from the c-*fos* gene promoter (sense strand, 5′-AGCTTCATTTCCCGTAAATCCCTA-3′) that binds activated Stat1 and Stat3 proteins (17). Protein-DNA complexes were resolved by nondenaturing PAGE and detected by autoradiography. For controls, STAT activity was determined in matched specimens obtained from nontumor tissues adjacent to the tumor, and also in NIH3T3 cells transformed by the Src oncoprotein (17) as an internal reference standard. Thus, levels of STAT activation were compared in nontumor and tumor tissues of the same patient, and normalization to the Src internal reference standard allowed comparison across different EMSA gels. Quantification of Stat3 activation levels was performed using ImageQuant software analysis of scanned EMSA gel bands. Anti-Stat1 and Stat3 polyclonal antibodies (Santa Cruz Biotechnology) were used to identify specific STAT family members. For use in supershift assays, 1 μl of the STAT antibodies was incubated with nuclear extracts for 20 min at room temperature before the addition of radiolabeled probe and electrophoresis.

Oligonucleotides and Transfections. The Stat3 antisense (5′-GCT CCA GCA TCT GCT GCT TC-3′) or control mismatch oligonucleotides (5′-GCT CCA ATA CCC GTT GCT TC-3′) were synthesized using phosphorothioate chemistry. To increase stability, oligonucleotides were synthesized with 2′-*O*-methoxyethyl modification of the five terminal nucleotides (underlined; Ref. 29). Transfections were carried out by the Lipofectamine-Plus method as described by the supplier (Life Technologies, Inc.). Briefly, DU145 cells were seeded at 2×10^6 cells/10-cm tissue-culture plates 18 h before transfection in RPMI 1640 supplemented with 10% FBS. Immediately before transfection, cells were washed once with PBS. Cells were transfected for 3 h with Lipofectamine-Plus (LF+) alone, with LF+/Stat3 antisense oligonucleotides, LF+/Stat3 mismatch oligonucleotides, or not transfected. The final concentration of the oligonucleotides was 250 nM. The transfection was terminated by aspirating the transfection medium, washing the cells one time with PBS and adding fresh RPMI 1640 containing 10% FBS. Another 24 h later, the nonadherent cells were washed off and the remaining cells were lysed EMSA or Western blot analysis.

Apoptosis and Proliferation Assays. For apoptosis assays, DU145 cells were treated for 24 h with Lipofectamine-Plus alone or with Stat3 antisense or Stat3 mismatch control oligonucleotides. Treated cells were stained with an antibody specific for activated caspase-3 using the supplier's protocol (Becton Dickinson PharMingen) and analyzed by flow cytometry. For proliferation assays, cell numbers were determined by counting with a hemocytometer using trypan blue exclusion.

Immunohistochemical Detection of Phospho-Stat3. Cytospins from LNCaP, DU145, and PC3 cells lines without treatment, LNCaP cells stimulated with IL-6 (10 ng/ml), and DU145 cells transfected with Lipofectamine-Plus (LF+) alone, with LF+/Stat3 antisense oligonucleotides, or LF+/Stat3 mismatch oligonucleotides were fixed in 95% ethanol for 10 min. Forty-five pairs of matched primary prostate tumors with normal tissue were fixed in 10% neutral-buffered formalin and embedded in serial 3–4-mm paraffin blocks. Five-μm thick sections were stained with H&E for histological examination. Representative histological sections and cytospins were then immunostained to localize Stat3. Immunostaining for phospho-Stat3 was performed using a rabbit antihuman polyclonal antibody (Phospho-Tyr705-Stat3; Cell Signaling, Beverly, MA). As negative controls, rabbit immunoglobulins (Vector, Burlingame, CA) were used to replace primary antibody. The 5-μm thick tissue sections described above were deparaffinized and hydrated in deionized water. The immunohistochemical staining was performed manually at room temperature, using the avidin-biotin-peroxidase complex method (Vectastatin Elite ABC kit; Vector Lab). Briefly, pretreatment for antigen retrieval with a pressure cooker involved heating cytospins and tissue sections with a microwave oven, in 250 ml of unmasking solution (Vector Lab) for 20 min at high-power level, followed by 20 min of cooling time. Slides were then treated with 0.025% trypsin in 50 mM Tris (pH 7.6) for 5 min at 37°C without prewarming. Endogenous peroxidase and nonspecific background staining were blocked by incubating slides with 3% hydrogen peroxide for 10 min. After washing with PBS for 5 min, slides were blocked with normal serum and 3% BSA for 10 min, followed by incubation with the phospho-Stat3 primary antibody, at a dilution of 1:400, overnight at 4°C. After rinsing with PBS for 5 min, slides were incubated with a biotinylated secondary antibody for 60 min and washed again. After washing with PBS for 5 min, slides were incubated with avidin-biotin complex for 1 h and washed again. Chromogen was developed with 3,3-diaminobenzidine (DAB Substrate kit for peroxidase; Vector Lab) or Nova-red (Nova-red Substrate kit for peroxidase; Vector Lab). All of the slides were lightly counterstained with hematoxylin for 30 s before dehydration and mounting. The pathologists (L. B. M., N. A.) evaluated immunohistochemical reactions in the cell lines and primary tumors.

Image Analysis Cytometry. The computer-assisted CAS-200 Image Analysis System was used to quantify immunohistochemical staining of positive nuclei/total number of cells (× 100 for percent). A minimum of 300 nonoverlapping and well-preserved cells were measured within at least 10 adjacent ×400 magnification fields in each sample. Quantification of nuclei positive for phospho-Stat3 staining was assessed in tumor and normal prostatic gland cells. The CAS-200 system operator was unaware of the Stat3 DNA-binding results by EMSA at the time of evaluation. Data were analyzed for statistical significance using the Wilcoxon signed-rank test.

RESULTS

Elevated Stat3 DNA-binding Activity in Human Primary Prostate Tumors. To determine whether constitutive activation of Stat3 is associated with prostate cancer *in vivo*, we performed EMSA analysis using nuclear extracts prepared from a series of 45 primary prostate tumor (*T*) specimens with matched adjacent nontumor (*N*) prostate tissues. As a positive control (+*C*) and internal reference standard for comparison across EMSA gels, identical aliquots of nuclear extracts were used from NIH3T3 cells transformed by the Src oncoprotein that have been shown to contain high levels of Stat3 activation (17–20). Quantitative pairwise comparative analysis of EMSA results (Fig. 1) revealed elevated Stat3 DNA-binding activity, to different extents, in 82% (37 of 45) of primary adenocarcinoma specimens examined (*Lanes T1* through *T45*) relative to matched adjacent nontumor tissues

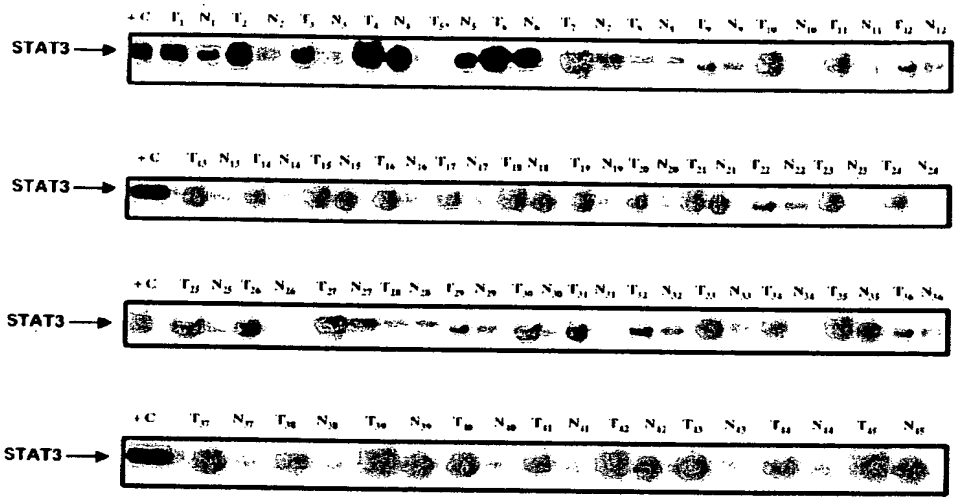

Fig. 1. Elevated Stat3 DNA-binding activity in human primary prostate tumors. EMSA analysis was performed using nuclear extracts prepared from 45 representative sets of matched primary adenocarcinoma and adjacent nontumor tissue specimens (*T*, primary tumors; *N*, matched nontumor tissues adjacent to primary tumors) using the radiolabeled hSIE probe, which binds activated Stat3 with high affinity. Results demonstrate elevated Stat3 activities in 37 of 45 tumor specimens compared pairwise with matched nontumor tissues. The positive control (*+C*) consisted of NIH3T3 cells transformed by the Src oncoprotein with high levels of constitutive Stat3 activation, which provided an internal reference standard for comparison across EMSA gels. *, this is the only case (*T5*) in which Stat3 activity was not detectable in tumor tissue.

(*Lanes N1* through *N45*). It is also notable that 29% (13 of 45) of adjacent nontumor tissues exhibited Stat3 DNA-binding activity higher than basal levels present in a majority of nontumor prostate tissues. Only one case displayed no detectable Stat3 activity in the tumor tissue (*Lane T5**). Thus, a high frequency of constitutive Stat3 activation is associated with primary prostate tumors *in vivo*, consistent with a role for activated Stat3 signaling in prostate cancer.

Analysis of Stat3 DNA-binding Activity in Human Prostate Cancer Cell Lines. To evaluate Stat3 activation in model prostate cancer cell lines, we performed EMSA analysis with the three well-characterized human prostate cancer cell lines DU145, PC3, and LNCaP. As shown in Fig. 2*A*, Stat3 DNA-binding activity is present in nuclear extracts prepared from all three of the prostate cancer cell lines examined. DU145 cells have the highest level, whereas LNCaP cells have the lowest level and are the least malignant, in agreement with the earlier findings of Ni *et al.* (30). Antibodies to Stat3, but not Stat1, could block and supershift this DNA-binding activity, confirming that the activity corresponds to Stat3 in all three cell lines (data not shown). We examined whether IL-6 could induce further Stat3 DNA-binding activity in the LNCaP cells. Treatment of serum-starved LNCaP cells with 10 ng/ml IL-6 induced a time-dependent activation of Stat3 (Fig. 2*B*). Antibodies to Stat3, but not Stat1, blocked this DNA-binding activity, confirming that it corresponds to Stat3. Consistent with the cell line data, antibody supershift analysis of all 45 patient tumor specimens from Fig. 1 demonstrated that the DNA-binding activity corresponds to Stat3 and not Stat1 (Fig. 2*C*, and data not shown).

Detection of Activated Phospho-Stat3 by Immunostaining of Human Prostate Cancer Cell Lines and Primary Tumors. To optimize the immunohistochemical protocol, we performed immunostaining with phospho-Stat3 antibody on cytospins with the model prostate cancer cell lines DU145, PC3, and LNCaP. This antibody reacts with the phosphorylated tyrosine 705 residue that is indicative of Stat3 activation (7, 8). As shown in Fig. 3*A*, phospho-Stat3 is detected in the nucleus with different intensities in all three of the human prostate cancer cell lines examined. The intensity of red stain (Nova-Red) corresponds to the level of active phospho-Stat3, whereas

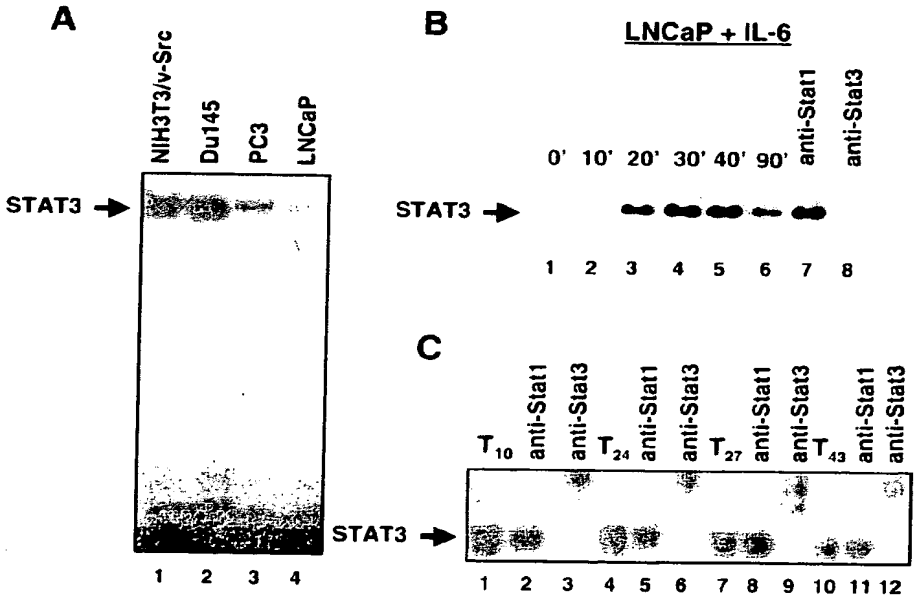

Fig. 2. Constitutive activation of Stat3 in model human prostate carcinoma cell lines. *A*, EMSA analysis of Stat3 DNA-binding activity in nuclear extracts prepared from human prostatic cancer cell lines using the radiolabeled hSIE probe. LNCaP is the least malignant cell line, and it was found to have the lowest Stat3 DNA-binding activity. NIH3T3 cells transformed by the Src oncoprotein serve as a positive control for constitutive Stat3 activation. *B*, kinetics of Stat3 activation by IL-6 in LNCaP cells. Cells were serum-starved to reduce the basal levels of Stat3 activation before stimulation with 10 ng/ml IL-6 for the indicated times (*0 min, 10 min, 20 min, 30 min, 40 min, 90 min*. Stat3 DNA-binding activity was positively confirmed by supershift analysis with antibodies to Stat3 (*Lane 8*) but not Stat1 (*Lane 7*). *C*, supershift analysis of representative primary tumor specimens from Fig. 1 demonstrates that the DNA-binding activities correspond to Stat3 and not Stat1. Similar results were obtained with all 45 of the patient tumor tissue specimens (data not shown).

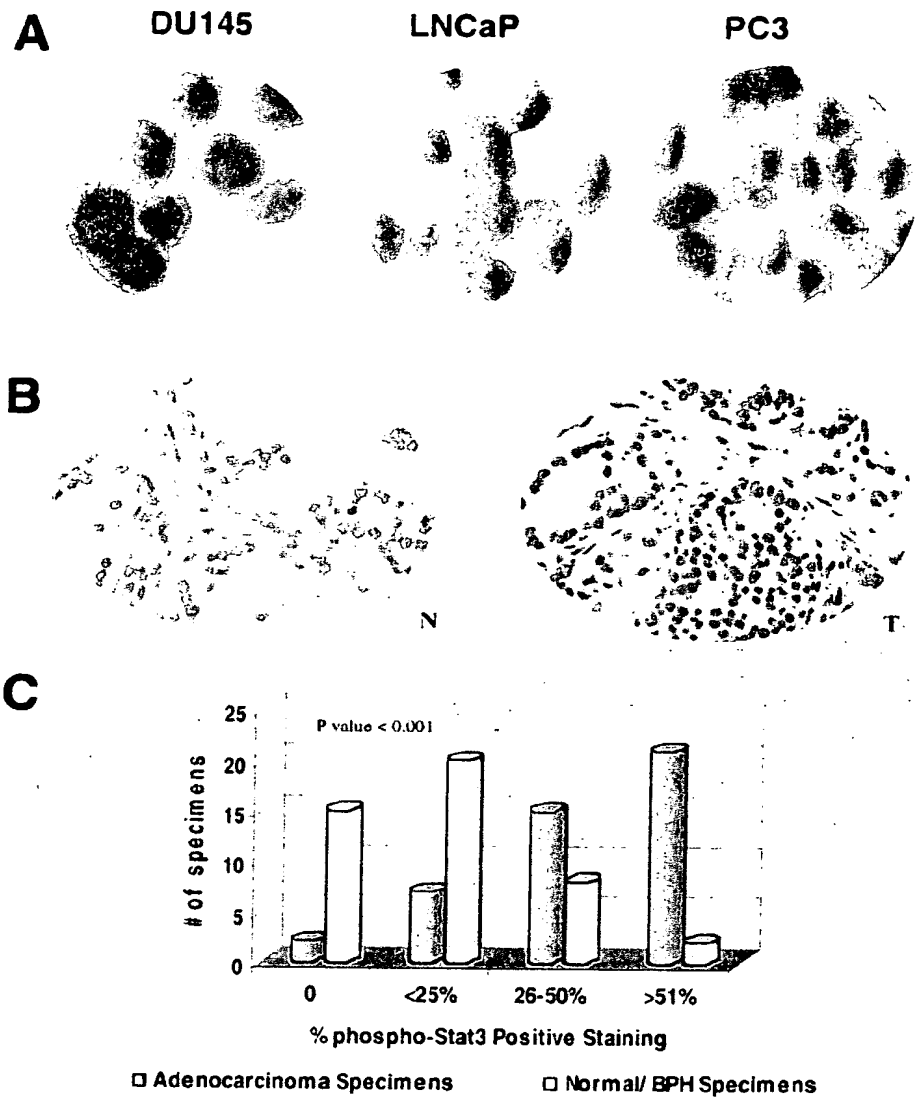

Fig. 3. Immunohistochemical detection of activated phospho-Stat3 protein in prostate cancer cells and primary tumors. *A*, cytospins from untreated DU145, PC3, and LNCaP cells show different intensities of antibody immunoreactivity (specific nuclear localization) that correlates with the Stat3 DNA-binding activity (compare with Fig. 2*A*). ×1000, oil immersion. *B*, representative sample of matched nontumor (*N*) prostate tissue with benign hyperplasia and prostate adenocarcinoma (*T*) tissue photographed at ×400. *C*, prostate specimens were stratified into four groups after quantification of phospho-Stat3 immunohistochemical staining of epithelial cells by computer-assisted imaging. Results are presented as % of phospho-Stat3 positively stained nuclei. The difference between the combined tumor and nontumor staining results is highly statistically significant (Wilcoxon signed-rank test, $P < 0.001$).

the blue stain (hematoxylin) is the counterstain. The levels of phospho-Stat3 immunoreactivity correlate well with the levels of Stat3 DNA-binding activity as measured by EMSA in these cells (compare Figs. 2*A* and 3*A*), with DU145 cells having the highest and LNCaP cells having the lowest levels.

The specificity of the immunoreaction for phospho-Stat3 was confirmed by specific ablation of Stat3 protein expression using antisense Stat3 oligonucleotides (see Fig. 4 below). After optimization of the immunohistochemical protocol, we used the phospho-Stat3 antibody to investigate the levels and cytological locations of activated Stat3 in the tumor specimens from prostate carcinoma patients. Immunohistochemistry demonstrated that low levels of nuclear phosphorylated Stat3 are restricted to the basal epithelial cells in normal (*N*) tissue adjacent to tumor tissue (Fig. 3*B*). In contrast, elevated levels of phospho-Stat3 are detected primarily in the nuclei of epithelial tumor cells in tumor (*T*) specimens (Fig. 3*B*). To quantify these observations, epithelial cells within the glands of prostate samples were analyzed by computer-assisted image analysis and the data are summarized in Fig. 3*C*. Statistically, the difference in phospho-Stat3 levels between the malignant specimens and nontumor specimens was highly significant ($P < 0.001$) using the Wilcoxon signed-rank test. Increased Stat3 tyrosine phosphorylation in the tumor cells detected by immunohistochemistry correlates well with elevated Stat3-DNA binding activities as detected by EMSA for these specimens (Fig. 1, and data not shown).

Elevated Levels of Phospho-Stat3 Correlate with High Gleason Score in Prostate Tumor Specimens. The levels of activated phospho-Stat3 protein in prostate tumors as detected by immunohistochemistry were compared with the clinicopathological characteristics of 45 prostate cancer patients. As summarized in Table 1, increased phospho-Stat3 levels were highly correlated ($P < 0.007$) with more malignant tumor specimens exhibiting Gleason scores ≥ 7. By contrast, there was no significant correlation between levels of phospho-Stat3 and either initial serum PSA levels or clinical stage (Table 1). It was not possible to assess the correlation between levels of phospho-Stat3 and established parameters of disease progression (*e.g.*, PSA levels after prostatectomy) because of the short time of follow-up after treatment of this cohort of patients. Thus, higher phospho-Stat3 levels correlate well with

Table 1 *Statistical correlation between levels of immunohistochemical phospho-Stat3 staining and clinicopathological characteristics of 45 patients with prostate adenocarcinomas*

Tumor characteristics	Phospho-Stat3 positive staining[a] 0% *n* (%)	≤25% *n* (%)	25–50% *n* (%)	≥51% *n* (%)	*P*
Initial serum PSA[b] (ng/ml)					0.62
0–4		1 (2)	3 (7)		
4.1–10	2 (4)	5 (11)	10 (22)	9 (20)	
10.1–15		1 (2)	1 (2)	6 (14)	
15.1 ≤20				7 (16)	
Clinical stage					0.50
T_2	2 (4)	4 (8)	8 (18)	18 (42)	
T_3	0	3 (7)	6 (13)	4 (8)	
Total Gleason score					0.007
≤6	2 (4)	3 (7)	8 (18)	5 (11)	
≥7		4 (8)	7 (16)	16 (36)	

[a] Percentage of nuclei that are phospho-Stat3 positive in primary prostate tumor specimens. *n* = number of patients; (%) = percentage of total patients.
[b] PSA, prostate-specific antigen.

more aggressive disease in prostate cancer as determined by Gleason score at the time of radical prostatectomy.

Antisense Stat3 Oligonucleotides Block Stat3 Protein Expression and DNA-binding Activity in Prostate Cancer Cells. To determine the consequences of down-modulating Stat3 protein expression in the model prostate cells, we transfected DU145 cells with Stat3 antisense oligonucleotide as described previously (31). Twenty-four h after transfection, EMSA was performed to measure levels of constitutive Stat3 DNA-binding activity. Fig. 4*A*, shows greatly decreased Stat3 DNA-binding activity in the *DU145 cells transfected with Stat3 antisense oligo (Lane 3)* compared with nontransfected cells (*Lane 1*), Lipofectamine-Plus (LF+) transfection reagent alone (*Lane 2*), or Stat3 mismatch control oligonucleotide (*Lane 4*). Antibodies to Stat3 could block and supershift this DNA-binding activity, confirming that the activity corresponds to Stat3. Western blot analysis of total Stat3 protein levels (Fig. 4*B*) demonstrates that Stat3 antisense (*Lane 3*) but not mismatch oligonucleotides (*Lane 2*) were able to substantially diminish Stat3 protein expression.

To establish that the immunohistochemical assay described above (Fig. 3) is specific for phospho-Stat3, we blocked Stat3 expression using antisense Stat3 oligonucleotide. This block in Stat3 expression should ablate specifically the phospho-Stat3 antigen and, thus, represents a highly rigorous control for antibody specificity. Immunostaining of cytospins prepared from DU145 cells treated with Stat3 antisense oligonucleotide demonstrated that the phospho-Stat3 staining was abolished (Fig. 4*C*). In addition, stimulation of LNCaP cells with IL-6 resulted in increased phospho-Stat3 immunostaining, as expected (Fig. 4*D*). These results demonstrate that the antibody and immunohistochemical methods used are specific for phospho-Stat3 and that the immunostaining results correlate well with Stat3 DNA-binding activities as measured by EMSA analysis.

Blocking Stat3 Signaling Inhibits Growth and Survival of Prostate Cancer Cells. Fig. 5*A* shows that treatment of DU145 cells with LF+/Stat3 antisense oligonucleotide induced significant growth inhibition that correlated well with the inactivation of Stat3 as measured by EMSA. The control mismatch oligonucleotide had an intermediate effect on cell growth, consistent with the partial effect on Stat3 DNA-binding activity (compare Figs. 4*A* and 5*A*). This intermediate effect of the mismatch oligonucleotide may reflect nonspecific toxicity. To determine whether down-modulation of Stat3 by using antisense oligonucleotide could induce apoptosis in prostate tumor cells, we transfected DU145 cells and, 12 h later, measured apoptosis using an early marker for apoptosis, caspase 3 activation (Fig. 5*B*). Results demonstrate a 3-fold increase in apoptosis of the cells treated with Stat3 antisense oligonucleotide compared with the Lipofectamine-Plus (LF+) reagent alone or Stat3 mismatch control oligonucleotide. Collectively, these findings indicate that activated Stat3 signaling is required for the growth and survival of prostate cancer cells.

DISCUSSION

In this study, we investigated whether constitutive activation of Stat3 protein is associated with prostate cancer *in vivo*. Significantly, EMSA analysis using nuclear extracts prepared from 45 primary prostate adenocarcinomas revealed elevated Stat3 DNA-binding activity in 82% of the tumor specimens compared with matched adjacent nontumor prostate tissues. These findings were confirmed by an independent measure of Stat3 activation based on immunohistochemical detection of phospho-Stat3. This immunohistochemical assay is useful for histological localization and quantification of activated Stat3 in primary tumor specimens. In addition, model human prostate cancer cell lines displayed constitutive activation of Stat3 DNA-binding activity, which could be further induced by IL-6 or blocked by antisense oligonucleotides specific for Stat3. Importantly, direct inhibition of Stat3 signaling was accompanied by growth inhibition and induction of apoptosis in prostate cancer cells.

Several lines of evidence suggest that IL-6 that are produced by tumor cells themselves or by adjacent stromal cells can modulate the growth of prostate tumor cells in an autocrine or paracrine manner (16, 32–34). The expression of IL-6 and its receptor has been detected not only in human prostate cancer cell lines but, more significantly, in human prostate carcinoma and benign prostate hyperplasia (BPH) specimens obtained directly from patients (35, 36). In addition, levels of IL-6 are elevated in the serum of patients with hormone-refractory and metastatic prostate carcinoma (35, 36). These findings suggest that the elevated Stat3 activation levels that we observed in prostate tumor tissues may arise from autocrine and/or paracrine stimulation by IL-6. Furthermore, it is possible that the elevated Stat3 activation detected in some of the adjacent nontumor tissues could also be induced by IL-6. Consistent with this possibility, levels of phospho-Stat3 were generally higher in nontumor tissues that were closer to the regions of tumor than in those that were farther away, suggesting that factors such as IL-6 secreted by tumor cells may induce Stat3 activation in adjacent tissues.[5]

Our findings are consistent with recent studies showing that IL-6 stimulates prostate cancer cell growth and activation of Stat3 signaling (37) and that the inhibition of Stat3 signaling blocks the growth of prostate cancer cells (30). In contrast, other studies (38, 39) suggested that Stat3 signaling correlated with IL-6-induced growth arrest and differentiation of prostate cancer cells. The apparent discrepancy between the latter (38, 39) and the former studies (30, 37) as well as the present study may be explained by other factors, such as androgens, which could modulate responses to Stat3 signaling. In this context, it may be significant that IL-6 undergoes a transition from being growth inhibitory to being growth stimulatory during progression to hormone-refractory prostate cancer (40). Moreover, recent studies indicate that IL-6 induces androgen receptor-mediated gene regulation through the Stat3 protein (41), raising the possibility that IL-6-induced Stat3 activation may contribute to the development of hormone-refractory prostate cancer. Consistent with a growth-stimulatory role of Stat3 in prostate cancer (30, 37, 41), our results demonstrate that the direct inhibition of Stat3 signaling by antisense Stat3 oligonucleotides blocks the growth and survival of these cells.

Our findings reported here are also in agreement with recent studies showing that phospho-Stat3 levels are elevated in malignant prostate

[5] L. B. Mora, unpublished observations.

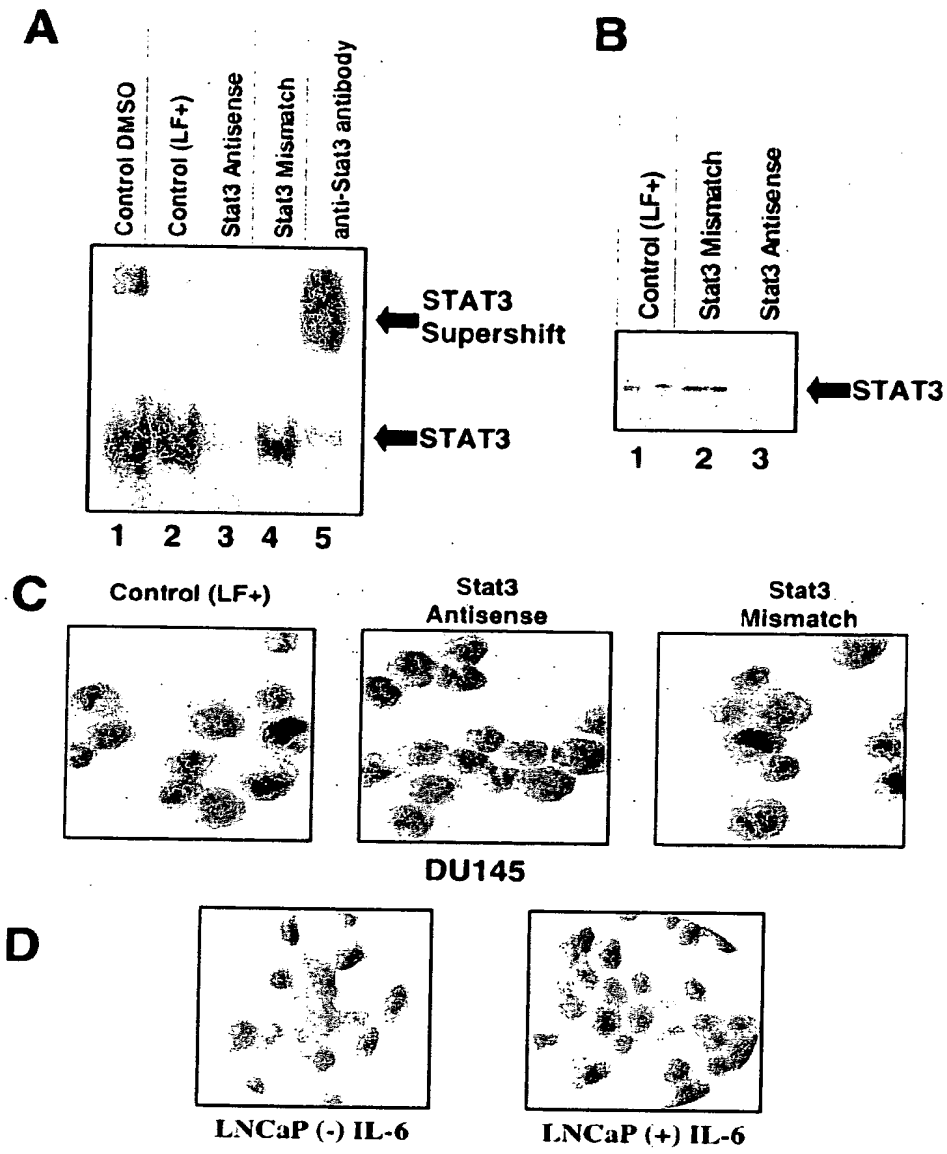

Fig. 4. Antisense Stat3 oligonucleotides block Stat3 protein expression, DNA-binding activity and immunohistochemical reactivity. *A*, Stat3 DNA-binding activity as measured by EMSA in nuclear extracts prepared from DU145 cells treated as follows: *Lane 1*, untreated control; *Lane 2*, Lipofectamine-Plus (*LF+*) reagent alone; *Lane 3*, LF+/Stat3 antisense oligonucleotide (250 nM); *Lane 4*, LF+/Stat3 mismatch oligonucleotide (250 nM); and *Lane 5*, untreated cells with anti-Stat3 antibody (*Supershift*). *B*, Western blot analysis of total Stat3 protein expression after treatment with: *Lane 1*, Lipofectamine-Plus (*LF+*) alone; *Lane 2*, LF+/Stat3 mismatch oligonucleotide; and *Lane 3*, LF+/Stat3 antisense oligonucleotide. *C*, immunohistochemical staining of phospho-Stat3 (×600) using cytospins from DU145 cells treated with Lipofectamine-Plus (*LF+*) alone, LF+/Stat3 antisense oligonucleotide, or LF+/Stat3 mismatch oligonucleotide. Different intensities of antibody immunoreactivity (specific nuclear localization) correlated with the Stat3 DNA-binding activities shown in *A*. The cells transfected with Stat3 antisense oligonucleotides had a completely negative nuclear immunoreactivity; demonstrating the specificity of the phospho-Stat3 immunohistochemical assay. *D*, immunohistochemical demonstration of induction of Stat3 activation in LNCaP cells after treatment with IL-6 (10 ng/ml at 40 min). ×400.

cells *in vivo* (42), further supporting a role for activated Stat3 in prostate cancer. To our knowledge, this is the first study in which the levels of activated Stat3 have been evaluated in parallel by a biochemical assay for Stat3 DNA-binding activity and an immunohistochemical assay for phospho-Stat3 in human tumors. Furthermore, levels of Stat3 activation were evaluated in the context of clinicopathological characteristics in a group of 45 patients with primary prostate adenocarcinoma. Our analysis demonstrates that higher levels of Stat3 activation are associated with higher Gleason scores (≥7), which is indicative of more aggressive and poorly differentiated tumors. This observation may be valuable for identification and management of high-risk prostate cancer patients, especially those with high Gleason scores. Additional studies are necessary to evaluate the prognostic significance of Stat3 activation in prostate cancer patients and correlation with other prognostic markers, such as Ki-67, angiogenesis and DNA ploidy, as well as clinical outcome by follow-up serum PSA levels (1–5).

Earlier studies showed that activated Stat3 signaling contributes to the growth and survival of diverse human cancer cells (24) such as multiple myeloma (15), breast carcinoma (43), melanoma (44), and head and neck squamous carcinoma (45). Thus, the development of therapeutic agents that target Stat3 signaling represents a potentially new treatment approach for prostate cancer and other human tumors (46). With the determination of phospho-Stat3 levels by immunohistochemistry, it might be possible to identify and stratify patients who may respond to therapies specifically targeting Stat3 signaling. This immunohistochemical assay has certain advantages over EMSA including cost effectiveness, reproducibility, and adaptability to the clinical setting. Importantly, the specificity of this immunohistochemical assay was confirmed by specifically ablating Stat3 expression with the use of antisense Stat3 oligonucleotides. Another major advantage is that the specific cell types (*i.e.*, tumor and nontumor cells) that have activated Stat3 can be distinguished by immunohistochemical but not by molecular approaches such as EMSA or Western blot analysis. Moreover, inexpensive and rapid follow-up of clinical outcome and response to treatment can be performed based on repeated

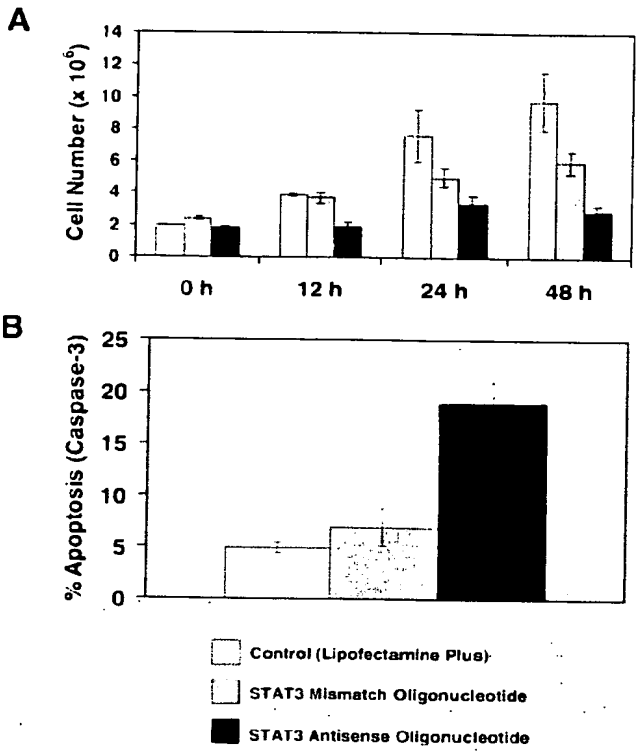

Fig. 5. Transfection with antisense Stat3 oligonucleotides induces growth inhibition and apoptosis of DU145 cells. *A*, DU145 prostate cancer cells were transfected with Stat3 antisense or mismatch oligonucleotides (250 nM) for 48 h and viable cell numbers were estimated by counting with a hemocytometer using trypan blue dye exclusion. *B*, apoptosis was assayed by flow cytometry using antibodies to activated caspase-3 after 12-h transfection with Stat3 antisense oligonucleotides. All values are expressed as means ± SDs ($n = 3$).

immunohistochemical assays using limited amounts of tissue material including core biopsies. It is important to note, however, that rapid processing (less than 15 min from surgical removal) is essential for preserving the *in vivo* phosphorylation state of Stat3 protein and, thus, for obtaining reliable data on Stat3 activation in tumor specimens. The immunohistochemical protocol is also critical to expose the epitope without denaturing it too much so that it can be recognized by the antibodies. In conclusion, these results encourage further exploration of the potential prognostic and therapeutic value of elevated Stat3 activation in patients with prostate carcinoma and other tumors.

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Links

PA-1, a human cell model for multistage carcinogenesis: oncogenes and other factors.

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We have developed a cell system which utilizes the human teratocarcinoma cell line PA-1, from which we have characterized four stages of tumor progression. Soon after establishment in culture PA-1 cells revert and are no longer tumorigenic in athymic nude mice. Later, PA-1 cells as they are passaged in culture, become tumorigenic at passage 100. The transition from nontumorigenic to tumorigenic is the result of the biological effects of an activated N-ras oncogene and can be reproduced by transfection of the cloned oncogene into preneoplastic PA-1 cells. Certain preneoplastic cells (prior to passage 100) in this series are susceptible to transformation by single oncogenes while others are not. In studying the basis of this susceptibility to single oncogene induced transformation we have found that somatic cell hybrids between preneoplastic cells which can suppress ras-induced transformation and ras-transformed cells are non-tumorigenic. Therefore, we believe that the progression from ras suppressing to ras susceptibility may be due to the inactivation of a trans-dominant suppressor gene. Our system has identified at least three steps which lead to tumorigenicity; establishment of growth past senesence, activation of a ras oncogene, and inactivation of an oncogene suppressor function. Further genetic alterations are necessary for tumor dissemination and metastasis.

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This article is part of a series on Gene therapy for carcinoma of the breast , edited by David Curiel.

Review

Gene therapy for carcinoma of the breast: Therapeutic genetic correction strategies

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Abstract

Gene therapy is a therapeutic approach that is designed to correct specific molecular defects that contribute to the cause or progression of cancer. Genes that are mutated or deleted in cancers include the cancer susceptibility genes *p53* and *BRCA1*. Because mutational inactivation of gene function is specific to tumor cells in these settings, cancer gene correction strategies may provide an opportunity for selective targeting without significant toxicity for normal nontumor cells. Both *p53* and *BRCA1* appear to inhibit cancer cells that lack mutations in these genes, suggesting that the so-called gene correction strategies may have broader potential than initially believed. Increasing knowledge of cancer genetics has identified these and other genes as potential targets for gene replacement therapy. Initial patient trials of *p53* and *BRCA1* gene therapy have provided some indications of potential efficacy, but have also identified areas of basic and clinical research that are needed before these approaches may be widely used in patient care.

Introduction

Because cancer is a somatic genetic disease, it should not be surprising that many gene therapy protocols have been designed to treat various forms of cancer. Even hereditary cancer syndromes generally include a required somatic gene alteration. For example, patients with hereditary breast and ovarian cancer due to *BRCA1* mutations are born with a mutation in one *BRCA1* allele, but only develop cancer after mutation or allelic loss of the other *BRCA1* allele. Thus, the complete genetic disorder is only present in the tumor cells that are targeted for somatic gene therapy. This is in contrast to inherited germline diseases, in which the genetic defect is present in all cells.

One strategy for cancer gene therapy is that of genetic correction. Because known gene defects are present exclusively in the cancer cells, genetic correction therapies provide a potential rational therapy that is molecularly targeted to cancer cells, but should not necessarily affect normal nonmalignant cells. A number of genetic correction strategies have been designed to treat cancer, including correction of *p53* and *BRCA1* [1*,2,3]. This review discusses some of the scientific information related to genetic correction strategies and then describes animal models and patient trials designed to initially test this therapeutic approach.

p53 gene therapy

p53 is the most commonly mutated tumor suppressor gene in solid tumors and is also mutated in the germline of patients with the rare hereditary Li-Fraumeni syndrome [4]. The *p53* gene is specifically relevant to the development and progression of breast cancer, because *p53* is frequently mutated in breast cancer specimens and Li-Fraumeni syndrome patients develop breast cancer as part of their multiple cancer syndrome [4]. Thus, *p53* genetic correction is a rational approach for breast cancer, particularly in those rare patients with breast cancer as part of Li-Fraumeni syndrome. The function of wild-type *p53* is suppression of cell proliferation through a multiprotein regulatory pathway that is focused around the retinoblastoma gene and control of apoptosis [5]. Because *p53* may naturally function as an inhibitor of cell proliferation, it inhibits cell growth in most normal and malignant cells [6,7], with few exceptions [8]. For this reason it may effectively inhibit tumor growth even in cancers that do not have *p53* mutations [6,7]. Preclinical animal studies of adenovirus-based *p53* gene therapy for cancer in both cell culture and animal models [9,10,11] have demonstrated tumor suppression. Clinical trials of *p53* gene therapy for lung cancer[1*,12*] have been reported, and these studies demonstrate gene transfer of adenoviral-p53, induction of apoptosis, and some indication of therapeutic response. In a phase 1 clinical trial of nonsmall cell lung cancer [12*], 8% of treated patients showed a partial response and 64% of patients showed disease stabilization ranging in duration from 2 to 14 months.

BRCA1 gene therapy

Although the molecular function of *BRCA1* is controversial and may include DNA repair or transcriptional functions [13], overexpression of *BRCA1* into sporadic breast or ovarian cancer cells, which usually show low *BRCA1* expression [14], results in growth inhibition and tumor suppression [15,16,17,18,19,20,21,22,23]. Growth inhibition by *BRCA1* has itself been controversial, leading some to question the rationale for *BRCA1* gene therapy [24], but subsequent studies have shown growth inhibition or tumor suppression by *BRCA1 in vitro* [16,17,19,20,21,22,23] and *in vivo* [15,17,21,25]. The mechanism of growth inhibition by *BRCA1* is unknown and may involve interactions with *WAF1/CIP1* [16], *p53* [17], *Rb* [23] or induction of apoptosis [18,19].

Although *BRCA1* is only mutated in a small percentage of breast or ovarian cancers, the majority of sporadic breast and ovarian cancers appear to express low levels of *BRCA1* messenger RNA and protein [14,26,27,28,29,30]. This appears to be a consequence of loss of heterozygosity and promoter methylation of the remaining *BRCA1* allele [14,26,27,28,29,30,31,32,33,34,35]. This finding is important because it indicates that restoration of normal 'wild-type' *BRCA1* expression levels in many sporadic cancers may inhibit tumors by a 'genetic correction' strategy, wherein the loss of *BRCA1* expression contributes to tumorigenesis. These results constitute the scientific basis for testing *BRCA1* gene therapy in patients with sporadic breast, ovarian, and prostate cancers that lack specific point mutations in the *BRCA1* gene.

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Several different *BRCA1* viral vectors have been constructed and tested for efficacy in preclinical xenograft models of breast and ovarian cancer. Initial studies of a *BRCA1* retroviral vector employed a complementary DNA that encoded a splice variant vector that eliminates the first 71 amino acids of the human protein, termed BRCA1sv [2,25,36*]. Studies of both growth inhibition and DNA repair do not identify cellular or molecular differences between *BRCA1sv* and *BRCA1* complementary DNAs [36*,37,38,39,40]. Intaperitoneal injection of either BRCA1sv or a full-length BRCA1 retroviral vector into ovarian cancer or breast cancer xenografts in nude mice produces tumor inhibition [2,15,25,36*,37,38,39]. These studies show that treatment of established SKOV3 or PA-1 ovarian cancer nude mice xenografts with either the full-length or the splice variant *BRCA1* retroviral vector results in tumor suppression. Necropsies showed that PA-1 tumor-bearing mice treated with control media or low-dose LXSN-*BRCA1sv* died with large intra-abdominal tumors and ascites, whereas mice with high-dose LXSN-*BRCA1sv* treatments died of lung metastasis with significantly smaller abdominal tumor. The PA-1 tumor model is an established xenograft model for ovarian cancer [37,38,39,41,42].

The published phase 1 trial of *BRCA1sv* retroviral gene therapy [2] demonstrated gene transfer and expression of the intraperitoneally injected LXSN-*BRCA1sv* vector. The vector was moderately stable in the peritoneum of these patients, and antibody formation was rare. The phase 2 trial performed in a group of patients with lower tumor burdens [36*], however, demonstrated that tumor size and immune status strongly influence patient response to retroviral vectors, and that vectors packaged in mouse cells are not sufficiently stable to treat patients with small volume intraperitoneal ovarian cancer.

Patient model systems for breast and ovarian cancer

The initial patient trials of *p53* and *BRCA1* gene therapy demonstrated the potential for gene therapy, but also clearly demonstrated both basic and clinical research that is needed before these therapies can be successful in patients. Initial human clinical trials of *BRCA1* retroviral gene therapy taught us that the approach was safe, but that healthy patients developed immune responses towards retroviral vector therapy that decreased vector stability and presumably prevented response. Based on this prior experience, we have redesigned our current human trials to employ a more immune-resistant MFG-based retroviral vector that is packaged in human producer cells. These trials should demonstrate whether this new generation of human cell-produced retroviral vector is more stable in the healthier patients with small volume disease. Because breast and ovarian cancers exhibit significant biologic and genetic similarities, gene therapies may be initially tested in the setting of ovarian cancer, employing peritoneal injection, and then ultimately tested in a more relevant breast cancer human disease model. Both types of patient model will be described in the following discussion.

The model system of metastatic ovarian cancer growing within a confined anatomic space (often bathed in ascitic fluid) has several advantages in safety and efficacy for studies of gene transfer into solid tumors. The pathology of metastasis into peritoneal-lined spaces often consists of small tumor implants with extravasation of tumor cells into the surrounding space, potentially allowing a reservoir for delivering gene transfer vectors to malignant cells. This pattern of spread is in contrast to other solid tumors such as breast, lung or melanoma that grow as solid three-dimensional masses anatomically located in sites that are inaccessible to currently available vector systems. Ovarian cancer provides a model system in which regional therapy (intraperitoneal infusion) could be curative in a reasonable percentage of cases. We have reported [2,36*] that the uptake and expression of the vectors can be readily assessed in this model system because direct access to the peritoneal cavity is possible through an implantable peritoneal catheter.

Because present gene therapy tools preclude systemic treatment strategies, we are initiating a regional treatment protocol to test the safety and potential efficacy of a human producer cell-derived MFG-*BRCA1* gene therapy by studying the effect of *BRCA1* retroviral gene transfer into breast cancer tumors that have recurred on the chest wall. The study population will consist of women who have failed one course of standard therapy and have biopsy-proved metastatic breast cancer involving the chest wall. Nodular chest wall disease constitutes a particular pattern of metastatic or recurrent breast cancer that is frequently resistant to treatment with standard therapy. We have selected chest wall recurrence of breast cancer for this study because of accessibility of the tumor both for vector administration and for biopsy and biologic analysis. Chest wall nodules will be biopsied and then injected daily for 4 days with MFG-*BRCA1* viral vector. The tumors will then be excisionally biopsied 1-4 weeks later, so that molecular and cellular studies of gene transfer, expression, and immune response can be evaluated. Because these nodules are often multiple, we will inject paired nodules with a placebo retroviral vector MFG in order to test directly whether observed effects are gene related or merely nonspecific effects of retroviral vector injection in humans. Immunologic and molecular studies will be performed on blood samples and tissue samples to evaluate immune response and vector pharmacokinetics, as in the ovarian cancer trial.

Conclusion

Genetic correction strategies are presently being developed and tested in animal models for human malignancies and in early patient trials. The cancer susceptibility genes *p53* and *BRCA1* have been tested in lung cancer and ovarian cancer patients, respectively, and have shown some potential for this antitumor strategy. *p53* gene therapy may be effective even against tumors that lack *p53* mutations, because *p53* may function as a growth inhibitor in a variety of gene transfer settings. The observation that sporadic breast and ovarian cancers show decreased *BRCA1* expression indicates that *BRCA1* gene therapy may be effective even against sporadic breast or ovarian cancer without *BRCA1* gene mutations. Although initial approaches to human gene therapy focused on germline inherited diseases, it has become evident that somatic genetic diseases like cancer represent appropriate targets for somatic gene therapy.

The clinical application of gene correction therapy will require advances in both basic science and clinical research. Key problems at present include the degradation of vector by the immune system and a need for higher levels of gene transduction. Solutions will require the development of improved vectors, improved vector delivery systems, and the fine-tuning of human gene therapy in appropriate models of human cancer.

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